Untersuchungen zur Lipoxygenase-vermittelten chemischen Verteidigung: Oxylipine aus Diatomeen und dem Moos *Physcomitrella patens*

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1. Einleitung

1.1 Das pelagische Nahrungsnetz

Populationen innerhalb einer Lebensgemeinschaft sind durch ein Netzwerk von Interaktionen miteinander verbunden. Diese Interaktionen sind häufig trophischer Natur und zeichnen sich durch „Fressen“ und „Gefressenwerden“ aus (Lampert & Sommer 1999).

In der einfachen Vorstellung einer aquatischen Nahrungskette lassen sich entsprechend des Energie- und Stofftransports vier trophische Ebenen charakterisieren: Phytoplankton (Primärproduzent)[1] > Zooplankton (Primärkonsument oder Sekundärproduzent) > planktivore Fische (Sekundärkonsument) und Raubfische (**Abb. 1**) (Hardy 1959). Während diese vertikalen Verbindungen innerhalb einer Lebensgemeinschaft Räuber-Beute Beziehungen darstellen, werden horizontale Interaktionen innerhalb einer trophischen Ebene häufig als Konkurrenzbeziehungen diskutiert. Abgesehen von extrem artenarmen Lebensräumen ist dieses Bild von Nahrungsketten jedoch zu einfach (Lampert & Sommer 1999). Herbivore Zooplankter selektieren zum Beispiel ihre Nahrung stärker nach Größe als nach ihrer trophischen Rolle. Ernährt sich der Kopepode *Calanus* von einem Phytoplankter, nimmt er die Stellung eines Primärkonsumenten ein; ernährt er sich dagegen von einem Phytoplankton fressenden Zooflagellat ist er ein Sekundärkonsument. An die Stelle des klassischen Konzepts, nach dem marine trophische Interaktionen als eine einfache pyramidale Nahrungskette aufgefasst werden, tritt daher die Vorstellung eines Nahrungsnetzes („The ocean´s food web: a changing paradigm“; Pomeroy 1974), das umso feinmaschiger erscheint, je genauer die inter- und intraspezifischen Wechselbeziehungen einzelner Organismen untersucht werden (Lavigne 1996, Link 2002).

Das Phytoplankton, bestehend aus phototrophen Organismen, bildet das Fundament aquatischer Nahrungsketten. Es fixiert 45 % des globalen Gesamt-Kohlenstoffs als phototrophe Organismen in organischer Materie (Legendre 1990, Smetacek 1999, Munn 2004) und bildet somit den Ausgangspunkt des marinen Energieflusses. Das Phytoplankton ist keine systematische Einheit, sondern wird funktionell definiert und seine Organismen sind gekennzeichnet durch die Fähigkeit zur sauerstoffbildenden Photosynthese. Es umfasst im Sinne der modernen Systematik Prokaryonten (Cyanobakterien und Prochlorobakterien) und pflanzliche Protisten (z. B.: Chromophyta, Dinophyta oder Raphidophyta) (Sommer 1998). Häufig wird das Phytoplankton nach seiner Größe in Pikoplankton (z.B.: Cyanobakterien; 0,2 - 2 µm), Nanoplankton (z.B.: Chrysophyceen; 2 - 20 µm), Mikroplankton (z.B.: Dinophyta: 20 - 200 µm) und Mesoplankton (z.B.: koloniebildende Phytoplankter: 200 µm - 2000 µm) eingeteilt.

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[1]**Primärproduzent**: Autotropher Organismus, **Sekundärproduzent**: Heterotropher Organismus, der organische Substanz assimiliert und einen Teil davon verbraucht.

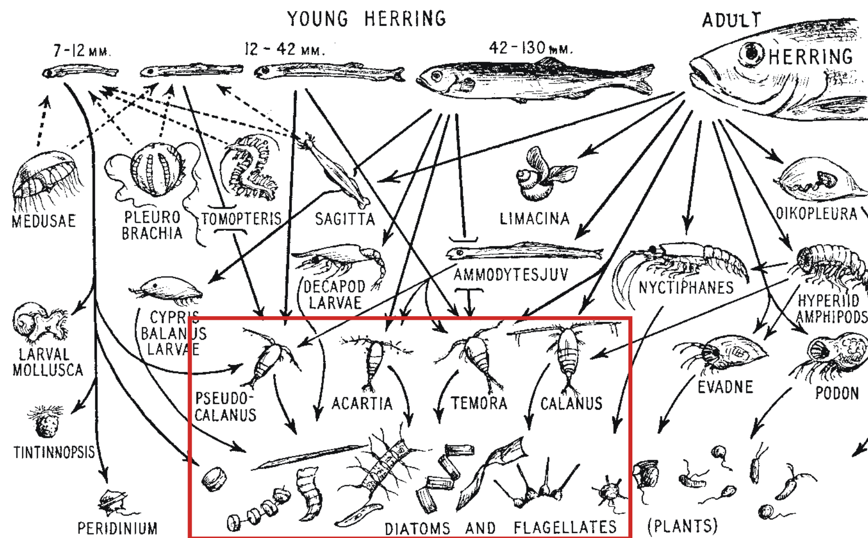

Abb. 1: Klassische Nahrungskette im Nordwest Atlantik nach Hardy (1956). In der graphischen Darstellung sind Räuber-Beute Beziehungen vertikal dargestellt und Konkurrenzbeziehungen horizontal. Die Umrandung hebt die in dieser Arbeit untersuchten marinen Organismen hervor.

Alle weiteren Organismen des Plankton sind Konsumenten und werden je nach Stellung innerhalb des Ökosystems beispielsweise als Herbivoren (= Pflanzenfresser), Karnivoren (= Tierfresser), Omnivoren (= Allesfresser) oder Detritivoren (= Aufnahme von organischen Sinkstoffen) bezeichnet (Sommer 1998). Mikroben und partikulärem organischem Material wurde jahrzehntelang nur eine unbedeutende Rolle in der Nahrungskette zugeschrieben. Erst von Azam *et al.* (1983) wurde diese Gruppe neben dem „Netz"-Phytoplankton (Zellgröße > 60 µm) als eine der wichtigsten Nährstoffquellen für marine Nahrungsketten identifiziert und als „*microbial loop*" bezeichnet (*microbial* bezieht sich in diesem Zusammenhang auf Organismen der Zellgröße des Pico- und Nanoplankton (Munn 2004)).

1.2 Untersuchung trophischer Interaktionen

Zahlreiche Studien wenden mathematische Modelle an, um trophische Interaktionen in planktonischen Systemen zu simulieren oder zu analysieren (Edwards & Yool 2000, Edwards *et al.* 2000, deYoung *et al.* 2004). In global angelegten Untersuchungen wird die Biodiversität des Phytoplanktons und Zooplanktons diskutiert (Ohman & Hirche 2001, Irigoien *et al.* 2004). Ein anderer Ansatz fokussiert auf Schlüsselorganismen des Nahrungsnetzes (Elser & Hessen 2005) und untersucht experimentell die spezifischen Interaktionen ausgewählter Räuber-Beute-Paare. Eine intensiv untersuchte Interaktion im marinen Ökosystem ist die Beziehung zwischen Kieselalgen (Diatomeen; Bacillariophyceae, **Abb. 2A-D**) und Ruderfußkrebsen (Copepoda, **Abb. 2E, F**).

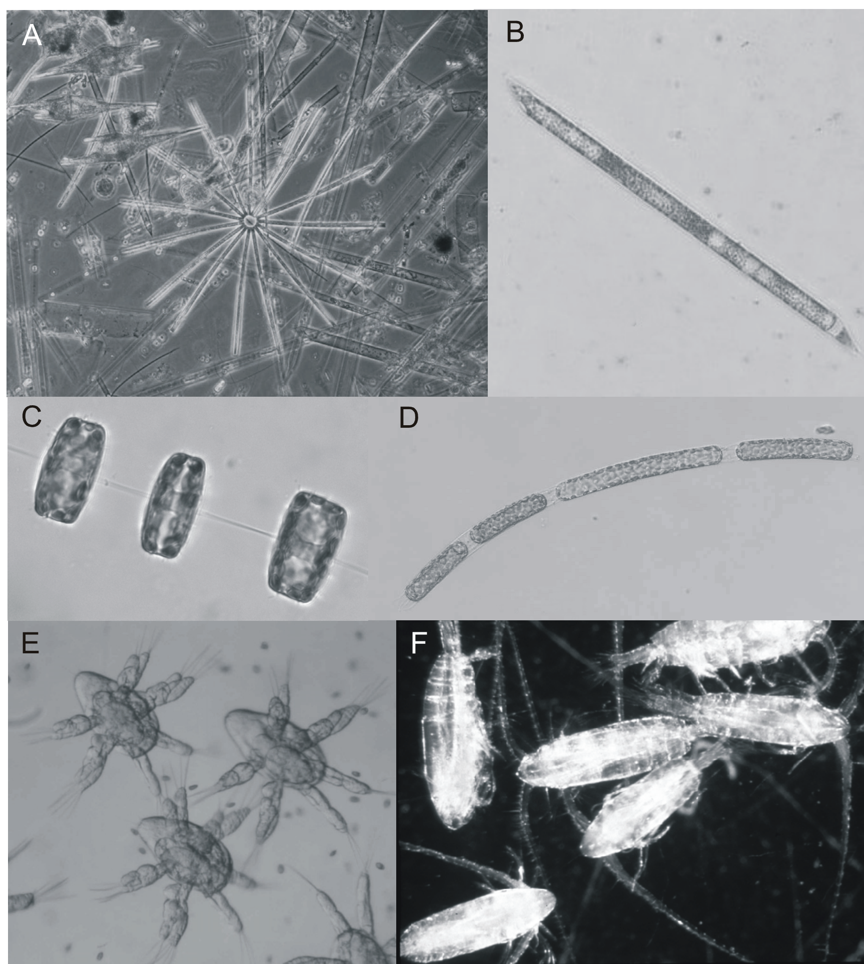

Abb. 2: (A) Phytoplankton Probe (Früjahrsblüte: Nord-Adria Station 1; Ianora *et al.* 2004); (B) *Rhizosolenia steigera*, (C) *Thalassiosira rotula*, (D) *Stephanopyxis turris*, (E) *Calanus helgolandicus*: Nauplius-Larven, (F) *Calanus helgolandicus*: adulte Kopepoden (**Abb**.: E und F mit freundlicher Genehmigung von Dr. Serge Poulet).

Copepoda ist eine Unterklasse der Crustacea (Krebse) und gehört den Arthropoda an. Die Kopepoden durchlaufen während ihrer Ontogenese die Stadien der Nauplius-Larven

(**Abb. 2E**) und der Copepodiden bevor sie zum adulten Tier heranwachsen (**Abb. 2F**) Während des Entwicklungszyklus verschiebt sich die Ernährungsweise von der Herbivorie (Nauplius-Larve) zur Omnivorie (adultes Tier). Da nur wenige Nauplius-Larven die adulte Lebensform erreichen, sind sie die zahlenmäßig vorherrschende Lebensform mehrzelliger Zooplankter im Mesoplankton.

Während die Prädation von Diatomeen durch Kopepoden an vielen Beispielen hinsichtlich Erkennung (Poulet 1976), Selektion (Marshall 1973, Poulet & Chanut 1975, Sykes & Huntley 1987, Cowles *et al.* 1988, Turner & Tester 1989), Fang (Poulet 1983, Hansen *et al.* 1994), Aufnahme (Paffenhöfer 1982, Strickler 1985) und Verdauung (Mayzaud 1986, Mayzaud *et al.* 1992) intensiv erforscht worden ist, waren zum Zeitpunkt des Beginns dieser Arbeit die chemischen Interaktionen bis auf wenige Ausnahmen in der Planktonökologie unverstanden (Wolfe 2000).

Chemisch analytische Methoden (Schreier 1984, Boland *et al.* 1992, Tholl *et al.* 2006), die zur Entschlüsselung trophischer Interaktionen terrestrischer Pflanzen (Baldwin 1988, Turlings *et al.* 1990, Arimura *et al.* 2000, Arimura *et al.* 2005) entscheidend beigetragen haben, stehen aufgrund der erschwerten Probennahme auf dem offenen Meer häufig nicht zur Verfügung, was die Identifizierung und Überwachung von neuen Verteidigungs-metaboliten oder Infochemikalien[2] häufig ausschliesst. Dennoch ist der Einfluss von Verteidigungs- oder Signalmetaboliten (z. B.: Kairomonen (Loose *et al.* 1993, von Elert & Pohnert 2000)) auf die intra- und interspezifischen Interaktionen demonstriert, so dass man eine regulierende Wirkung auf den Energietransfer zwischen verschiedenen Trophiestufen vermuten darf (Hay 1996, Wolfe 2000, Zimmer & Butman 2000, Winder 2002).

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[2]Terminologie (Dicke & Sabelis 1988): **Infochemikalien:** Eine Chemikalie, die in der natürlichen Umgebung die Interaktion zwischen zwei Individuen fördert und eine Antwort des Empfängers evoziert. **Pheromon:** Eine Infochemikalie, die die Interaktion zwischen zwei Individuen der gleichen Art vermittelt, wobei das aussendende Individuum oder beide profitieren. **Allelochemikalie:** Eine Infochemikalie, die die Interaktion zwischen zwei Individuen vermittelt, die zu verschiedenen Arten gehören. Je nachdem ob der Sender, der Empfänger oder beide profitieren wird zwischen Allomonen, Kairomonen und Synomonen unterschieden.

1.3 Phytoplankton/Zooplankton Interaktion

Planktologen, Oceanographen, Biochemiker und chemische Analytiker verständigten sich auf einem Kolloquium über Diatomeen/Kopepoden-Interaktionen in marinen Ökosystemen darauf, dass nur durch einen engen interdisziplinären Ansatz die vielfältigen chemisch-vermittelten Interaktionen von Organismen verschiedener trophischer Ebenen richtig entschlüsselt und interpretiert werden können (**Publikation 1**). Um mit hoher zeitlicher und räumlicher Auflösung die Wirkung von Metaboliten auf Konsumenten zu bestimmen, sind Freilandversuche notwendig, die durch eine empfindliche chemische Analytik begleitet werden müssen. Aufgrund des Artenreichtums des Phytoplanktons und der komplexen Nahrungsselektion durch die Kopepoden (Dam *et al.* 1995, Turner *et al.* 2001), ist eine chemische Analyse des gesamten Nahrungsspektrums, das von Kopepoden aufgenommen wird und einen Größenbereich von 10 µm - 200 µm umfasst, sinnvoll (**Publikation 2**). Die Untersuchung und Analytik von ausgewählten Spezies des Phytoplanktons ist dagegen eine zu starke Vereinfachung (Ban *et al.* 1997, d'Ippolito *et al.* 2002a, d'Ippolito *et al.* 2002b, Pohnert *et al.* 2002), um die vielfältigen Interaktionen zwischen Beute und Konsument entschlüsseln zu können. Um zu ermessen, welche ökologisch relevanten Konzentrationen potenzieller Verteidigungsmetabolite oder Infochemikalien das Wachstum, die Reproduktion oder das Überleben der Konsumenten beeinflussen können, ist eine robuste *in situ* Analytik notwendig, die reaktive Metabolite in der komplexen Matrix des Phytoplanktons durch Derivatisierungen stabilisiert (**Publikation 2**) bevor sie analysiert werden. Die Entwicklung dieser Methoden ist eine Vorraussetzung für ein tieferes Verständnis der durch Verteidigungsmetaboliten und Infochemikalien beeinflussten Planktonökologie und somit ein Schwerpunkt der vorliegenden Arbeit (**Publikationen 2, 13, 14**). Für das Verständnis der Interaktionen trophischer Ebenen ist nicht nur der reaktive Metabolit selbst interessant, sondern darüber hinaus auch seine Biosynthese (**Publikation 4**) und deren Regulation (**Publikation 14**).

Die Interaktion zwischen Phytoplankton und Zooplankton wurde von Harvey *et al.* (1935) erstmalig systematisch im Englischen Kanal untersucht und ist bis heute von großem Interesse geblieben (Irigoien *et al.* 2004). Dies erklärt sich zum einen durch den hohen Anteil des Phytoplanktons an der globalen Photosyntheseaktivität (Field *et al.* 1998); zum anderen bilden planktonische Kopepoden die größte Gruppe innerhalb der Metazoen und beeinflussen als sekundäre Produzenten direkt pelagische Fischpopulationen (Mann 1993, Ohman & Hirche 2001).

Die Diatomeen bilden eine der größten Gruppen innerhalb des Phytoplanktons und sind eine schon seit langem intensiv untersuchte Klasse (Fleming 1939), deren Sukzession und Zusammensetzung in vielen marinen Gewässern eingehend studiert wurde (z. B.: Kieler Bucht: (Smetacek 1975, Reinheimer 1991); Bretagne: (Grall 1972, Gailhard *et al.* 2002);

Southern California Bight: (Goodman *et al.* 1984); Dabob Bay: (Frost 1991)). Im Jahresgang der Phytoplanktonsukzession bilden Diatomeen oft Frühjahrs- oder Sommerblüten aus. Während dieser Periode dominieren einige wenige Diatomeenarten das Phytoplankton. Diese Blüten unterscheiden sich von den „*harmful algal blooms*“ (HAB), die in Küstengewässern von Flagellaten dominiert werden (Hallegraff 1993, Sournia 1995). Die Chlorophyllkonzentration steigt während einer Diatomeenblüte um mindestens eine Größenordnung an (Smetacek 1999). Eine solche planktonische Algenblüte entwickelt sich, wenn die Wachstumsrate der Blüten bildenden Art die Summe aller reduzierenden Faktoren übersteigt. Prädation durch Mikrozooplankton (z. B.: heterotrophe Protisten) (Tillmann 2004) und Mesozooplankton (z. B.: Kopepoden) (Frost 1991, Dam *et al.* 1993) werden häufig als entscheidende limitierende Faktoren angeführt. Inspiriert durch Flemming´s Analysen (1939, *„The Control of Diatom Populations“*) formulierte bereits Riley (1946, 1947) das erste mathematische Modell, das die Bedeutung der durch Diatomeen verursachten Frühjahrsblüten für die herbivoren Zooplankter herausstellt. Die Struktur dieser planktischen Zönosen wird von bottom-up (Hairston *et al.* 1960) und top-down (Odum 1969) Effekten beeinflusst (Lampert & Taylor 1985, Brett & Goldman 1997). Unter dem bottom-up Effekt, der die Entwicklung des Zooplanktons besonders stark beeinflusst, versteht man die wachstums- und reproduktionslimitierende Wirkung der Nahrung (produzentenreguliertes Modell), während die Prädation ein top-down Effekt ist (konsumentenreguliertes Modell) (Chase 2000). Carpenter *et al.* (1985) postulieren in einem Konsumenten regulierten Modell die Fortpflanzung derartiger Einflüsse (*„The Trophic Cascade“*), die von trophischer Ebene zu trophischer Ebene immer weiter nach unten vordringen. Gemäß dem produzentenregulierten Modell haben dagegen die Räuber den geringsten und die Primärproduzenten den größten Einfluss auf die Nahrungskette.

Wenn aufgrund von Ernährungsdefiziten die maximal mögliche Wachstumsrate (Richardson & Verheye 1999) von Primärproduzenten nicht erreicht werden kann, gilt das Wachstum als nahrungslimitiert. Ernährungsdefizite entstehen durch zu geringe Nahrungsmengen oder mangelhafte Nahrungsqualität. Da die Nahrungskette über den Energiefluss zwischen trophischen Ebenen definiert wurde und die meiste Energie in organischen Verbindungen gespeichert ist (Lindeman 1942, Morowitz 1968), wird die zur Verfügung stehende Nahrungsmenge häufig als Kohlenstoffkonzentration angegeben (Lampert 1977).

Dennoch kann auch unter resourcengesättigten Bedingungen produktiver Gewässer das Wachstum und/oder die Reproduktion des Zooplanktons eingeschränkt sein (Frost 1991, Moss *et al.* 1991, Boersma & Vijverberg 1994). Daher wird der Nahrungsqualität eine zunehmend größere Bedeutung beigemessen (Stottrup & Jensen 1990, Jonasdottir 1994, Ederington *et al.* 1995, Pond & Harris 1996, Muller-Navarra *et al.* 2000). Bis heute gibt es allerdings keinen allgemein akzeptierten Parameter, der die zahlreichen Facetten der

Nahrungsqualität für marine und limnische Zooplankter umfassend beschreibt (Brett & Goldman 1997). Folgende Aspekte sind in den letzten Jahrzehnten von Forschungsinteresse gewesen:

- Form und Größe der Nahrungspartikel (Porter 1973)
- Aufschluss und Verdaubarkeit der Nahrung (Van Donk *et al.* 1997, Smetacek 2001, Hamm *et al.* 2003)
- Limitierung in der Verfügbarkeit von Phosphor (Sommer 1992) und Stickstoff (Checkley 1980, Kiorboe 1989, Hessen *et al.* 1997)
- Limitierung in der Verfügbarkeit von Polyenfettsäuren (Jonasdottir 1994, Brett & Goldman 1997) und Sterolen (Hassett 2004)
- Limitierung in der Verfügbarkeit von Aminosäuren (Guisande *et al.* 2000) und Proteinen (Kleppel & Hazzard 2000)
- Toxizität der Nahrung (Turner & Tester 1997, Dutz 1998, Colin & Dam 2002)

Eine anorganische oder organische Substanz kann nahrungslimitierend sein, wenn sie essentiell ist und in geringeren Konzentrationen in der Nahrung vorkommt als im Konsumenten (Sterner *et al.* 1998). In zahlreichen Laborversuchen und Freilandstudien wurde gezeigt, dass dies für bestimmte vielfach ungesättigte Polyenfettsäuren in der phytoplanktonischen Nahrungsquelle limnischer und mariner Zooplankter zutrifft. Insbesondere die Eicosapentaensäure (EPA; 20:5ω3)[3], die Docosahexaensäure (DHA; 22:6ω3) sowie die Steroidonsäure (18:4ω3) erhöhen signifikant die Wachstumsrate (Brett & Goldman 1997, Müller-Navarra *et al.* 2000) oder den Reproduktionserfolg von Zooplanktern (Jonasdottir 1994, Lee *et al.* 1999, Shin *et al.* 2003).
Während sich die meisten Studien bei der Betrachtung der Phytoplankton/Herbivor Interaktion auf den Herbivor konzentrieren (Lampert & Sommer 1999, Irigoien *et al.* 2002, Irigoien & Harris 2003) haben Wolfe *et al.* (1997) und Smetacek (1999) den Blickwinkel auf Verteidigungsmechanismen des Phytoplanktons gelenkt, die als Fraßabwehr bzw. Fraßreduktion diskutiert werden (Smetacek 2001). Diese Mechanismen umfassen sowohl mechanische Verteidigungsstrategien aufgrund von Form und Stabilität z.B. silifizierter Diatomeenzellwände (Van Donk *et al.* 1997, Hamm & Rousseau 2003), als auch chemische Verteidigungsstrategien in der Planktonökologie, die zum Beispiel bei Dinoflagellaten (Turner

[3]**Nomenklatur der Fettsäuren**; Beispiel: 20:5ω3 gibt 20 die Zahl der Kohlenstoffatome der Fettsäure an (C20), 5 indiziert die Zahl der methylen überbrückten Doppelbindungen (n = 5), ω3 gibt die Position des Kohlenstoffatoms an, das die erste Doppelbindung gezählt vom Methylterminus enthält.

& Tester 1997), Cyanobakterien (Landsberg 2002) oder beim Nanoplankter *Emiliana huxleyi* untersucht worden sind (Wolfe 2000).

1.4 Chemische Verteidigung

Dinoflagellaten und Cyanobakterien können sich durch Massenerscheinen zu einer toxischen Phytoplanktonblüte akkumulieren. Je nach Spezies produzieren sie konstitutiv toxische Metaboliten, die zur Gruppe der Neurotoxine, wie Saxitoxin (*Alexandrium*) und Brevitoxin (*Gymnodinium*), oder zu den Hepatotoxinen wie Nodularin (*Nodularia spumigena*) gezählt werden. Die ökologische Bedeutung dieser Substanzen ist allerdings trotz intensiver Forschung weitestgehend unverstanden (Turner & Tester 1997), wobei darauf verwiesen sei, dass einige Kopepoden das von *Alexandrium* produzierte Saxitoxin akkumulieren können (Dutz 1998) und infolgedessen ihre filtrierenden Konsumenten (z.B.: Fische) mit den gespeicherten Toxinen kontaminieren (Graneli *et al.* 1990, Hansen *et al.* 1992, Van Dolah 2000, Landsberg 2002).

Der einzellige Nanoplankter *Emiliana huxleyi* setzt bei der Nahrungsaufnahme durch den Protozoen *Oxyrrhis marina* (Dinophycee) Dimethylsulfid (DMS) und Acrylsäure aus Dimethylsulfonpropansäure (DMSP) frei (Wolfe *et al.* 1994), da nach Zelllyse DMSP und das Enzym DMSP-Lyase gemischt werden. In „Choice-Experimenten" wurde gezeigt, dass *O. marina* sich bevorzugt von den Isolaten von *E. huxleyi* ernährt, die weniger Acrylsäure bilden (Wolfe & Steinke 1996) und eine niedrigere DMSP-Lyase Aktivität aufweisen (Wolfe *et al.* 1997). Wolfe *et al.* (2000) haben daher fraßaktivierte Verteidigungsmechanismen auf der Grundlage von freigesetzter Acrylsäure vorgeschlagen.

Dieser Mechanismus unterscheidet sich von der konstitutiven Produktion der Neurotoxine bei Dinoflagellaten und wird als aktivierbarer chemischer Verteidigungsmechanismus[4] bezeichnet. Ein aktivierbarer chemischer Verteidigungsmechanismus muss nach Paul & van Alstyne (1996) drei Kriterien erfüllen:

1. Ein biologisch inaktiver Metabolit wird in einen aktiven transformiert.
   Kleine Strukturveränderungen des Metaboliten erhöhen dabei signifikant die biologische Aktivität.
2. Der Prozess läuft innerhalb von wenigen Sekunden oder Minuten ab.
3. Die Transformation wird durch wenige Enzyme vermittelt.

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[4]Definitionen: **Konstitutive Verteidigung:** Die Verteidigung erfolgt durch Metabolite, die im Organismus gespeichert oder an der Oberfläche angereichert sind. **Induzierte Verteidigung:** Nach Rezeption von durch Fraßfeinden oder Pathogenen emittierten Signalen werden Verteidigungsmetabolite *de novo* synthetisiert. **Aktivierte Verteidigung:** Gespeicherte untoxische Substanzen werden nach Zellverletzung und Aufhebung der Enzym/Substrat Kompartimierung direkt in reaktive Folgeprodukte umgewandelt.

Neuere Experimente von Strom *et al.* (2003) haben gezeigt, dass weder DMS noch die Acrylsäure direkt als Verteidigungsmetabolit wirken, sondern möglicherweise nur ein Indikator für eine unzureichende Nahrungsqualität der Beute für den Herbivor darstellt. Zur abschließenden Klärung sind Fütterungsexperimente, bei denen die postulierten Verteidigungsmetaboliten durch *Mikro-Verkapselung* (Langdon *et al.* 1985) oder durch Imprägnierung neutraler Futterquellen eingebracht werden, notwendig.

Weil der individuelle Reproduktionserfolg die Grundlage für den evolutiven Erfolg einer jeden Art bildet, ist er beudeutsam für die Ausprägung von Nahrungsketten (Elser & Hessen 2005). Infolgedessen wird nicht nur die direkte Fraßhemmung sondern auch der Reproduktionserfolg als Kriterium eingesetzt, um den nachhaltigen Einfluss des Phytoplanktons auf die Herbivoren zu bewerten.
Der Reproduktionserfolg von Kopepoden wird im Allgemeinen nach der Eiablagerate (Eier Tag^{-1} $Weibchen^{-1}$ (Fekundität, *fecundity*) und nach dem Schlupferfolg der Larven aus den Eiern (*hatching success*) bemessen. Als idealer Modelorganismus hat sich der marine Kopepode *Calanus* zur Erforschung des Reproduktionserfolges erwiesen und wird dahingehend seit 60 Jahren untersucht (Marshall *et al.* 1953). *Calanus helgolandicus* ist ein nahezu unpigmentierter Kopepode und eignet sich somit zur *in vivo* Beobachtung der Gonadenentwicklung, der frühen Zellteilungen gelegter Eier und zur Untersuchung der Nauplius-Larven. Calanus ist zudem im Nordatlantik und Mittelmeer weit verbreitet und von großer ökologischer Bedeutung (Übersichtsartikel: Bonnet 2005). Calanus ernährt sich als Herbivor hauptsächlich von Phytoplankton (Bacillariophyceae: Diatomeen, Dinophyta: Dinoflagellaten) und Mikroplankton (Cyanobakterien) (Breteler *et al.* 1999), sowie in geringerem Ausmaß als Karnivor von Mikrozooplankton (Ciliata) (Ptacnik 2003, Ptacnik *et al.* 2004).
Im Allgemeinen stellen aber Diatomeen eine der Hauptnahrungsquellen für Kopepoden dar. Sie sind im Gegensatz zu anderen Phytoplanktern wie den Chlorophycen (Zhukova & Aizdaicher 1995, Breteler *et al.* 1999) reich an vielfach ungesättigten C20-Polyenfettsäuren (PUFA) (Ackman *et al.* 1968, Volkman *et al.* 1989, Dunstan *et al.* 1994, Zhukova & Aizdaicher 1995). Die PUFAs müssen entweder direkt mit der Nahrung aufgenommen oder aus den essentiellen C18-Polyenfettsäuren von den Kopepoden synthetisiert werden (Moreno *et al.* 1979). Da Diatomeen Eicosapentaensäure (EPA) und Arachidonsäure (AA) enthalten, wird ihnen eine hohe Nahrungsqualität zu geschrieben, die die Reproduktion von Kopepoden stimulieren kann (Kleppel 1993).

1.5 Ungesättigte Aldehyde als teratogene Substanzen

Poulet und Mitarbeiter (1994) beobachteten, dass eine mit der Diatomee *Thalassiosira rotula* angereicherte Diät bei calanoiden Kopepoden in Laborexperimenten sowohl niedrigere Eiablageraten als auch einen geringeren Schlupferfolg bedingt als eine Dinofalgellaten (*Prorocentrum minimum*) Diät. Applikation von Extrakten dieser Diatomeen zu frisch gelegten Eiern arretierte die Embryogenese. Diese Ergebnisse motivierten zahlreiche Laboratorien den Diatomeeneffekt auf insgesamt 16 verschiedene Kopepodenarten der nördlichen und südlichen Hemisphäre zu untersuchen. Die untersuchten 37 Diatomeen/Kopepoden Kombinationen wurden in vier Gruppen kategorisiert (Ban *et al.* 1997; Übersichtsartikel: Paffenhöfer 2002):

I. Sowohl Eiablage als auch der Schlupferfolg sind reduziert.
II. Nur der Schlupferfolg ist reduziert, nicht aber die Eiablage.
III. Die Eiablage ist reduziert, nicht aber der Schlupferfolg.
IV. Es wurde kein negativer Effekt beobachtet.

Lediglich eine untersuchte Diatomeenart zeigte keinen Effekt (Kategorie IV), während die meisten untersuchten Diatomeen / Kopepoden Paarungen in die Kategorie I und II gruppierten. Diese Beobachtungen führten zur der Annahme, dass Diatomeen eine schädliche Nahrungsquelle für verschiedene Kopepodenarten wie *Calanus helgolandicus* (Poulet *et al.* 1994), *Arcatia clausi* (Ianora *et al.* 1996) oder *Calanus pacificus* (Uye 1996) darstellen.

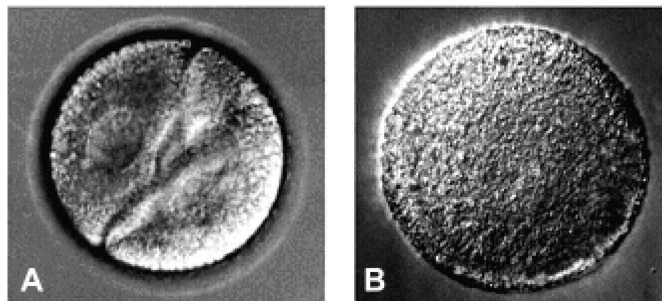

Abb. 3: Mikroskopische Fotographien eines normalen Embryos (**A**) (zwei Blastomeren Stadium; ø = 180 µm) produziert von einem Weibchen (*C. helgolandicus*), das sich von Dinoflagellaten ernährt hat und eines Embryos mit inhibierter Zellteilung (**B**) produziert von einem Weibchen, das sich von *T. rotula* ernährt hat (**Publikation 6**). In diesem Fall wird keine Nauplius-Larve schlüpfen (Kategorie I oder II).

Der „schädliche" Effekt der Diatomeen auf den Reproduktionserfolg ist dabei umso stärker, je höher die Konzentration der Diatomeen in der Nahrung ist und je länger die Fütterung andauert (Chaudron *et al.* 1996). Dieser Prozess ist reversibel, wenn die Diatomeen durch Dinoflagellaten substituiert werden (Laabir *et al.* 1995, Uye 1996). Die von Ban *et al.* (1997)

definierten verschiedenen Kategorien (I-III) wurden mit einer unterschiedlich intensiven Wirkung schädlicher Metaboliten zu erklären versucht (Lee *et al.* 1999).
Diese Ergebnisse haben zu verstärktem Forschungsinteresse und zu kontroversen Diskussionen geführt, die die Richtigkeit des experimentellen Aufbaus in Frage stellten. Der Diskurs kumulierte in einem Briefwechsel in der Zeitschrift MARINE ECOLOGY PROGRESS SERIES zwischen Jonasdottir *et al.* (1998) und Ianora *et al.* (1999), in dem Jonasdottir *et al.* (1998) die Experimente mit Diatomeenextrakten sowie Fütterungsexperimente mit hohen Zelldichten im Bereich von 10^4-10^7 Zellen ml^{-1} (Chaudron *et al.* 1996), pointiert kritisierte:

„[these] procedures are no more realistic than suffocating humans in chocolate sirup and the concluding chocolate is toxic".

Während ein Teil der wissenschaftlichen Gemeinschaft in Diatomeen eine Futterquelle hoher Qualität sieht (Jonasdottir & Kiorboe 1996) und Mono-Diäten (= ausschließlich Diatomeen als Nahrung) als unbalanciert adressieren (Jonasdottir 1994), postulierten Miralto *et al.* (1999b) eine von Diatomeen produzierte Substanz als Ursache embryonaler Missbildungen. Die hohen Zelldichten in Laborexperimenten (Chaudron *et al.* 1996) lassen sich unter Berücksichtigung der Tatsache rechtfertigen, dass Frühjahrsblüten, die von Diatomeen dominiert werden, ebenfalls eine hohe Zellkonzentration aufweisen (Legendre 1990).
In der Tat beobachteten Miralto und Mitarbeiter (1999) in der Adria während der Frühlingsblüte von *Skeletonema costatum* (Familie: Thalassiosiraceae) geringe *in situ* Schlupfraten von 12 % bei frisch gefangenen *C. helgolandicus*, während bei Kopepoden, die nach den Diatomeenblüten isoliert wurden, normale Schlupfraten von 90 % erreicht wurden. In einem Großansatz wurden Diatomeen-Extrakte von *Thalassiosira rotula*, die zur gleichen Familie wie *S. costatum* gehört, aufgearbeitet und daraus (2*E*,4*Z*,7*Z*)-Decatrienal, (2*E*,4*E*,7*Z*)-Decatrienal und (2*E*,4*E*)-Decadienal isoliert. Die Zugabe dieser $\alpha,\beta,\gamma,\delta$-ungesättigte Aldehyde (*polyunsaturated aldehydes;* PUA) zu frisch gelegten Eiern von *C. helgolandicus* bewirkt eine Arretierung der Embryogenese (Miralto *et al.* 1999a) ähnlich wie sie mit wässrigen Auszügen aus Diatomeen aber nicht mit Extrakten von Dinoflagellaten beobachtet worden ist (Poulet *et al.* 1994). Die von Diatomeen produzierten ungesättigten Aldehyde wurden somit als biologisch aktive Komponenten identifiziert, die eine antiproliferierende Wirkung haben und die Entwicklung der Kopepodeneier negativ beeinflussen (Buttino *et al.* 1999, Ianora *et al.* 1999, Miralto *et al.* 1999a,b). Diese Ergebnisse stellen das klassische Konzept in Frage, nach dem Diatomeenblüten eine essentielle Nahrungsquelle sind und gemäß dem bottom-up Modell das Wachstum und die Reproduktion der Herbivoren positiv beeinflussen. Dieser generalisierten Sichtweise widersprachen Irigoien *et al.* (2002) mit einer global angelegten Studie von

ressourcengesättigten Gewässern, in der sie belegten, dass die Vitalität und der Schlupferfolg der Kopepodeneier in den meisten Fällen nicht durch Diatomeen angereichertes Phytoplankton beeinträchtigt werden. Die sich scheinbar widersprechenden Beobachtungen (Miralto *et al.* 1999a, Irigoien *et al.* 2002) legen die Vermutung nahe, dass die Diatomeen/Kopepoden Interaktionen in verschiedenen Gewässern spezifisch betrachtet werden müssen. Des Weiteren haben auch Irigoien *et al.* (2002, *Supporting Information*) in 24 % der untersuchten Proben einen reduzierten *hatching success* (< 80%) festgestellt, diesen aber nicht weiter diskutiert. Weder isolierte Diatomeenarten noch das Phytoplankton selbst wurden von Irigoien *et al.* (2002) auf die Produktion von $\alpha,\beta,\gamma,\delta$-ungesättigten Aldehyden getestet.

Weiterhin ist der Einfluss potenziell schädlicher Algenblüten nicht allein auf die Zahl der gelegten Eier und den Schlupferfolg zu beschränken, sondern die nachfolgende Larvenentwicklung muss ebenfalls in Betracht gezogen werden, um einen vollständigen Überblick maternaler Effekte auf die Ontogenese zu erhalten (Alekseev & Lampert 2001, **Publikation 5, 14**).

Untersuchungen verschiedener Zönosen über einen längeren Zeitraum sind daher notwendig (z. B.: Küstengebiet: Bretagne, Frankreich (**Publikationen 12, 14**); Fjord: Dabob Bay, Washington, USA (**Publikation 13**); Upwelling system: Chile), um eine durch $\alpha,\beta,\gamma,\delta$-ungesättigte Aldehyde beeinflusste Interaktion zwischen Kopepoden und Diatomeen zu verstehen und zeitlich wie räumlich auflösen zu können. Um den potenziellen Einfluss der Aldehyde zu diskutieren, wurde ein erster Freilandversuch in der Dabob Bay (Washington, USA) in den Jahren 2002 und 2003 chemisch-analytisch begleitet (**Publikation 13**). Während der Phytoplanktonsukzession wurden Diatomeen isoliert und kultiviert, so dass eine Überprüfung des Potenzials einer chemischen Verteidigung durch die Aldehyde möglich wurde. Nach weiteren Verbesserungen der analytischen Methoden (**Publikation 2**) wurden die $\alpha,\beta,\gamma,\delta$-ungesättigten Aldehyde auch *in situ* gemessen und ihr Einfluss während der Diatomeensukzession auf den Reproduktionserfolg von *C. helgolandicus* an der marinen biologischen Station in Roscoff (Bretagne, Frankreich) in den Jahren 2003 und 2004 studiert. Das Gebiet 48°45´N und 3°58´W im westlichen englischen Kanal wurde zweimal wöchentlich beprobt (**Publikation 12,14**).

In marinen Ökosystemen wurden bis jetzt rund 10.000 Diatomeenarten beschrieben. Dennoch waren zu Beginn meiner Arbeit nur zwei marine Arten, *S. costatum* und *T. rotula*, als Produzenten von ungesättigten Aldehyden identifiziert (Miralto *et al.* 1999a, d'Ippolito *et al.* 2002a, d'Ippolito *et al.* 2002b) worden. In der Tat zeigte die Untersuchung verschiedener Diatomeenkulturen grundlegende Unterschiede in der Aldehydproduktion (Pohnert *et al.* 2002). Während das *T. rotula* -Isolat aus der Adria erwartungsgemäß als Aldehydproduzent identifiziert worden ist, setzt ein anderes Isolat derselben Art aus den Küstengewässern vor

Kalifornien keine Aldehyde frei (Pohnert *et al.* 2002). Vor diesem Hintergrund scheinen alle Deutungen der bei Diatomeendiäten beobachteten Reduktion des Fortpflanzungserfolges ohne weitere Bestimmung der Aldehydkonzentration in der Nahrung problematisch (Ceballos & Ianora 2003). Eine zu Fütterungsexperimenten simultan verlaufende Quantifizierung der Aldehydkonzentration ist daher vor dem Hintergrund der Ergebnisse von Pohnert *et al.* (2002) notwendig. Um einen Überblick über das tatsächliche toxische Potenzial verwendeter Diatomeen zu erhalten und um eine gezielte Versuchsplanung von Fütterungsexperimenten zu ermöglichen, ist eine Reihenuntersuchung sowohl ökologisch interessanter (Blüten bildende Arten) als auch klassischer „Labor-Diatomeen“ (z. B.: *Thalassiosira pseudonana*, *Thalassiosira weisflogii*, *Phaeodactylum tricornutum*) Voraussetzung (**Publikation 3**).

Die Kontroverse zwischen Jonasdottir *et al.* (1998) und Ianora *et al.* (1999) über den schädlichen Effekt der Diatomeen auf den Reproduktionserfolg der Kopepoden sowie die widersprüchlichen Interpretationen von Freilandversuchen (Miralto *et al.* 1999a, Irigoien *et al.* 2002) lassen sich nicht ohne eine begleitende *in situ*-Analytik der Nahrung auflösen. Im Rahmen dieser Arbeit wurde daher die analytische Methodik entwickelt, um zwischen den bestehenden widersprüchlichen Interpretationen verschiedener Forschergruppen vermitteln zu können (**Publikationen 1-3, 5, 7, 12-14**).

Ein weiterer Hauptkritikpunkt gegenüber den von Miralto *et al.* (1999a,b) durchgeführten Experimenten beruht auf den hohen (2*E*,4*E*)-Decadienal Konzentrationen (2 $\mu g\ ml^{-1}$), die notwendig waren, um die Embryogenese frisch gelegter Kopepodeneier vollständig zu arretieren. Im Hinblick auf die Situation im Ozean handelt es sich um eine unrealistisch hohe Konzentration (Hay 1996).

Trotz allem wurden diese Experimente auf den Seeigel (Echinodermata: *Paracentrotus lividus*) erfolgreich übertragen. *P. lividus* kann stimuliert werden, eine leicht zugängliche größere Menge an Eiern zu produzieren, mit denen Zelltoxizitätstests durchgeführt werden können (Buttino *et al.* 1999, Adolph *et al.* 2003). Eier, die mit Diatomeenextrakten behandelt worden sind, zeigten globuläres und in der Mitte der Zelle konzentriertes Chromatin. In einer vergleichenden Struktur/Wirkungsanalyse wurden die antiproliferierenden Wirkungen des (2*E*,4*Z*)-Decadienals, des (2*E*,4*E*)-Decadienals, des α,β-ungesättigten (2*E*)-Decenals und des gesättigten Decanals auf die frühen Zellteilungen des Seeigels miteinander verglichen (Adolph *et al.* 2003). Unabhängig von der Geometrie oder dem Fehlen der γ,δ-Doppelbindung beim (2*E*)-Decenal sind diese Moleküle in ähnlichen Konzentrationen biologisch wirksam (7 µM ≡ 50 % Inhibierung der ersten Zellteilung). Auch kurzkettige ungesättigte Aldehyde sind aktiv, so dass generell das Michael-Akzeptorsystem der $\alpha,\beta,(\gamma,\delta)$-ungesättigten Aldehyde als das strukturwirksame Prinzip angesehen werden kann. Decanal oder 2-Decanon, die nicht als Michael-Akzeptoren fungieren, wirken nur bei sehr hohen Konzentrationen oder überhaupt nicht (Adolph *et al.* 2003). Solche Michael-

Akzeptoren können *in vivo* mit nukleophilen Aminosäureresten von Lysin und Cystein (Schauensstein 1967) oder z.B. mit der DNS (Carvalho *et al.* 2000) reagieren.

Zur Quantifizierung können flüchtige Aldehyde in einem einfachen Verfahren mit Hilfe der Festphasenmikroextraktion (=*Solid Phase Microextraction*; SPME) (Louch *et al.* 1992, Maier *et al.* 1996, Spiteller & Spiteller 2000) aus dem abgeschlossenen Gasraum über einer konzentrierten Diatomeenkultur extrahiert und anschließend im Injektor eines Gaschromatographen, der mit einem Massenspektrometer verbunden ist, thermodesorbiert werden. Da die Varianz einzelner SPME relativ hoch ist und die ungesättigten Aldehyde mit der Diatomeenmatrix reagieren können, bevor sie in die Gasphase treten, wurde zur Verbesserung der Reproduzierbarkeit und zur Senkung der Nachweisgrenze eine Derivatisierungsmethode basierend auf der Reaktion von *O*–(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamin Hydrochlorid (PFBHA·HCl) mit Aldehyden entwickelt (**Abb. 4, Publikation 2**). Diese Reaktion läuft unter physiologischen Bedingungen (*in situ*) ab, so dass PFBHA·HCl direkt zur Kultur zugegeben werden kann, bevor die Aldehydbildung durch Ultraschallbehandlung aktiviert wird.

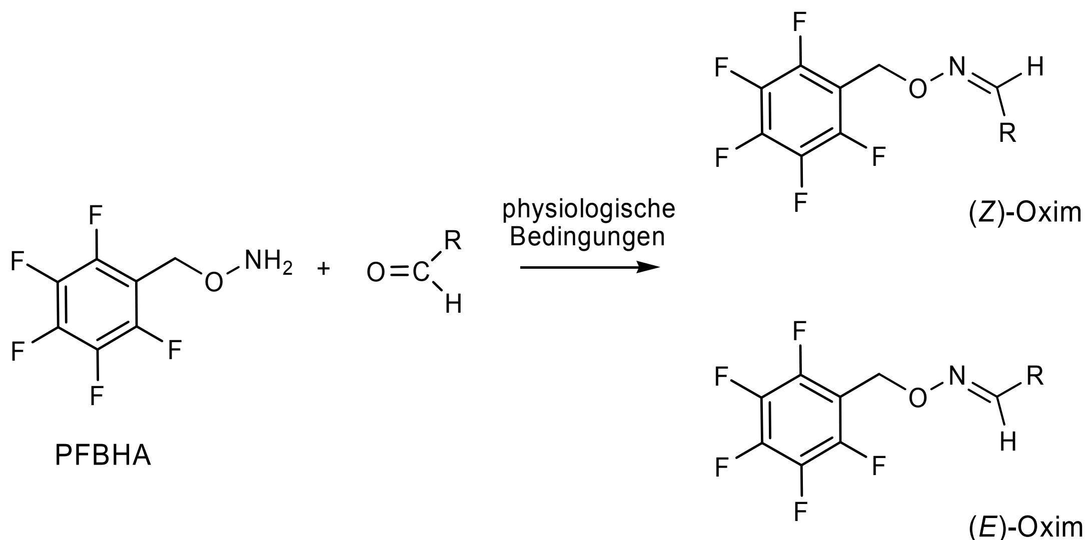

Abb. 4: Reaktion zwischen dem Derivatisierungsreagenz PFBHA und einem Aldehyd

1.6 Verwundungsaktivierte Verteidigung von Diatomeen

Durch die hohe Reaktivität der α,β,γ,δ-ungesättigten Aldehyde setzt sich die Zelle dem Risiko einer Selbstvergiftung aus (Carvalho *et al.* 2000). Die Kompartimierung von konstitutiv produzierten toxischen Substanzen (Fowden & Lea 1979, Baldwin & Callahan 1999) ist eine generelle Strategie dies zu vermeiden. Größere Organismen können spezialisierte Gewebe ausbilden, die toxische Substanzen bevorraten (Hefetz & Blum 1978).

Untersuchungen an intakten Kulturen von *T. rotula* zeigten, dass keine mit SPME nachweisbaren Mengen an ungesättigten Aldehyden (z.B.: Decatrienal) in die Umgebung freigesetzt werden. Auch lassen sich die Verteidigungsmetaboliten nicht extrahieren, wenn die gesamte Enzymaktivität durch Ansäuern unterdrückt wird (Pohnert 2000). Ausschließlich unter physiologischen Bedingungen werden nach Zellverwundung innerhalb von Sekunden größere Mengen flüchtiger Aldehyde (4,1 fmol $Zelle^{-1}$) produziert (Pohnert 2000).

Dieser Mechanismus wurde von Pohnert (2000) als aktivierte chemische Verteidigung bezeichnet, die sich nicht direkt gegen den Herbivoren richtet, sondern als teratogene Substanz auf die Entwicklung der Nachkommenschaft einwirkt (Miralto *et al.* 1999a). Da es sich bei der Aldehydfreisetzung um eine sehr schnelle wundinduzierte Antwort handelt, müssen die beteiligten Enzyme bereits translatiert und aktiv sein (Pohnert 2002).

Eine verwundungsaktivierte Verteidigungsstrategie von Einzellern scheint auf den ersten Blick wenig sinnvoll zu sein. Aufgrund hoher Bruttowachstumsraten durch vegetative Zellteilung von bis zu drei Verdoppelungen pro Tag (Sommer 1998), wird aber nur ein Teil der Population durch herbivore Zooplankter eliminiert (Wolfe 2000). Daher kann man spekulieren, dass die Gesamtpopulation der Diatomeenblüte von einer Reduktion der Individuenzahl der Zooplankter profitiert, auch wenn ein Teil von ihr gefressen wird (Wolfe 2000).

1.7 Biosynthese α,β,γ,δ,-ungesättigter Aldehyde

Die Struktur der Dienale legt nahe, dass es sich um fettsäureabgeleitete Metabolite handelt, die nach Oxidation von Polyenfettsäuren entstehen. Im Gegensatz zu der vielfach beschriebenen Autooxidation von Lipiden, die durch katalytische Mengen an Fe^{2+} oder Fe^{3+} verursacht wird (Frankel 1998, Spiteller & Spiteller 1998, Spiteller 2003), wird angenommen, dass im Falle der Diatomee *Gomphonema parvulum* Polyenfettsäuren enzymatisch mit Hilfe von Lipoxygenasen und Hydroperoxid-Lyasen in ungesättigte Fettsäuren mit einem α,β,γ,δ-ungesättigten Aldehydterminus umgesetzt werden (Pohnert & Boland 1996, Hombeck *et al.* 1999).

Während bei Tieren Polyenfettsäuren der Kettenlänge C20 die Vorläufersubstrate für die Biosynthese der Leukotriene und Prostaglandine darstellen (Maas *et al.* 1982, Brash *et al.*

1983, Yamamoto 1992, Rouzer & Marnett 2003), basieren die Lipoxygenase vermittelten Biosynthesen der Blütenpflanzen auf der Umsetzung von α-Linolensäure (= LEA) bzw. Linolsäure (= LA) (Feussner & Wasternack 2002). Die dabei gebildeten strukturell vielfältigen Phytooxylipine spielen eine wichtige Rolle bei der pflanzlichen Entwicklung (Hildebrand 1988) und der Stressantwort (z. B.: Wundhormon: Traumatin (Zimmermann & Coudron 1979) und Jasmonsäure (Wasternack & Parthier 1997)).

Höhere Pflanzen[5] enthalten bis auf wenige Ausnahmen (z. B.: *Agathis robusta*, Gymnosperma, Araucariacae; Wolff *et al.* 1999) fast ausschließlich Fettsäuren mit Kettenlängen von 16 oder 18 C-Atomen (**Abb. 5**), in zahlreichen Gruppen der niederen Pflanzen, wie den Algen, Farnen und Moosen, findet man allerdings auch solche mit 20 und 22 Kohlenstoffatomen und 4, 5 oder 6 Doppelbindungen. Rhodophyceae (Rotalgen; Zhukova & Aizdaicher 1995) und Phaeophyceae (Braunalgen; Smith & Harwood 1984) sowie Diatomeen (Kates & Volacni 1966) und Euglenophyceae (Augentierchen; Kates & Volacni 1966) können außerordentlich hohe Konzentrationen an Arachidon- und Eicosapentaensäure als Bestandteil ihrer Komplexlipide aufweisen (**Abb. 5**). Der Anteil an Arachidonsäure am Gesamtgehalt der Fettsäuren beträgt z.B. bei *Physcomitrella patens* bis zu 30 % (Grimsley *et al.* 1981, Mikami & Hartmann 2004). Arachidonsäure wird bei den Laubmoosen hauptsächlich in Phospholipiden gespeichert und ist an der Stressregulation beteiligt (Vandekerkhove *et al.* 1984, Hartmann *et al.* 1986, Euler 1987).

Es ist bemerkenswert, dass strukturähnliche fettsäureabgeleitete Aldehyde sowohl in höheren Pflanzen (Grosch 1968, 1976, Noordermeer *et al.* 2001), in Moosen (Dembitsky 1993), in Diatomeen (Wendel & Jüttner 1996, Pohnert *et al.* 2002), in Chlorophyta (Akakabe *et al.* 2003) und in Phaeophyceae (Boonprab *et al.* 2003a, Boonprab *et al.* 2003b) gefunden werden. Während höhere Pflanzen ausschließlich C18-Fettsäuren in Oxylipine transformieren können, werden bei Rotalgen vorzugsweise C20-Fettsäuren, bei Braunalgen C18- und C20-Fettsäuren (Gerwick 1993) sowie bei Diatomeen C16- und C20-Fettsäuren umgesetzt (Pohnert 2000, d'Ippolito *et al.* 2004). Da die Laubmoose mit ihrem Fettsäureprofil eine Zwischenstellung zwischen den Algen und den höheren Landpflanzen einnehmen (Asakawa 1986), sind sie ein interessantes Untersuchungsobjekt für die differentielle Transformation von C18- und C20-Fettsäuren durch Lipoxygenasen und Hydroperoxid-Lyasen (**Abb. 5**, **Publikationen 8-10**). Unter den Landpflanzen ist keine Lipoxygenase bekannt, deren bevorzugtes Substrat *in vivo* oder *in vitro* Arachidonsäure (AA), Eicosapentaensäure (EPA) oder Docosahexaensäure (DHA) ist.

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[5]Der Begriff „höhere Pflanzen“ ist beschränkt auf die Spermatophyten (Samenpflanzen). Den Samen bildenden Pflanzen sind die sich mit Sporen vermehrenden, blütenlosen Pflanzen gegenübergestellt, die als Kryptogamen oder „niedere Pflanzen“ bezeichnet werden.

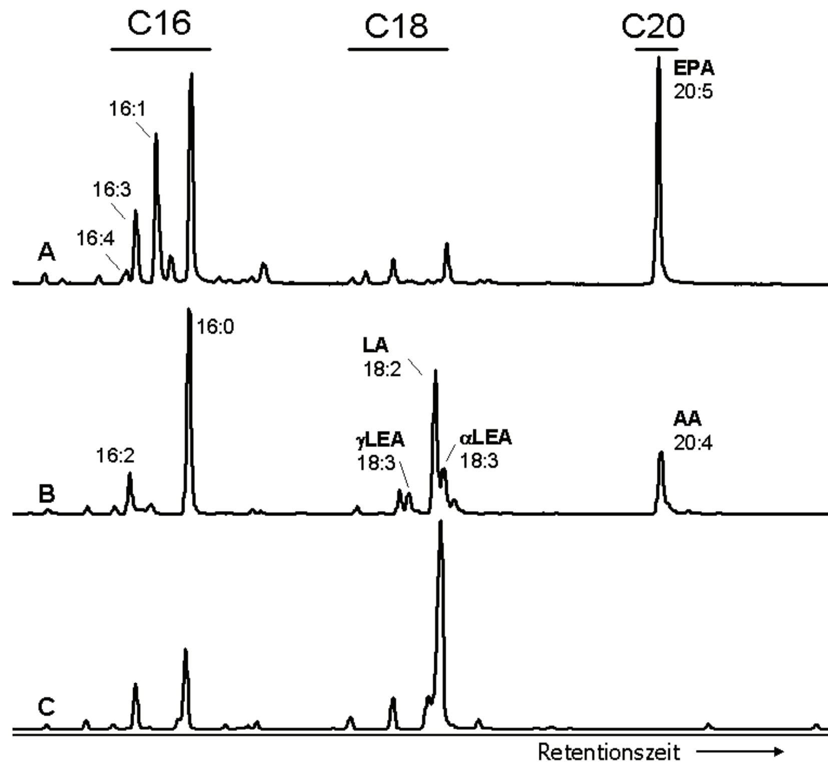

Abb. 5: Typische Gas-Flüssig-Chromatogramme methylierter Fettsäuren (**A**) der Diatomee *Stephanopyxsis turris*, (**B**) dem Moos *Physcomitrella patens* und (**C**) der höheren Pflanze *Arabidopsis thaliana* (Blatt) nach vollständiger Hydrolyse der Lipide (getrennt auf einer unpolaren EC5-Kapilliarsäule von Altech, USA); LA = Linolsäure, LEA = Linolensäure, AA = Arachidonsäure, EPA = Eicosapentaensäure

In Diatomeen wurde die erste Identifizierung biosynthetischer Vorstufen von Decadienal und Decatrienal im zellfreien System durchgeführt (Pohnert 2000). Hierzu wurden Zellen einer *T. rotula* Kultur durch Ultraschall zerstört und nach Abtrennung der Zellfragmente potenzielle Vorstufen zugesetzt, die zum Zweck der Identifizierung und Aufklärung der Biosysnthese stabil-isotopenmarkiert waren (Hombeck *et al.* 1999, Pohnert 2000), **Publikation 4**). Inkubation von kommerziell erhältlicher stabil-isotopenmarkierter [5,6,8,9,11,12,14,15-$^{2}H_{8}$]-Arachidonsäure ([$^{2}H_{8}$]-C20:4ω6; [$^{2}H_{8}$]-AA) weist die C20-Polyenfettsäure als exklusives Vorläufermolkül von Decadienal aus, da der C11-C20 Terminus der Fettsäure in den C10-Aldehyd eingebaut wird. Interessanterweise werden C18:4ω3 und C22:6ω3-Fettsäuren, die eine dem Decatrienal entsprechenden Terminius haben nicht als Substrat akzeptiert, sondern ausschließlich Eicosapentaensäure (C20:5ω3; EPA). Während hoch ungesättigte C20-Polyenfettsäuren die Vorläufermoleküle von Decadienal und Decatrienal sind, wird Octadienal aus einer 16:3ω4-Fettssäure gebildet (d'Ippolito *et al.* 2003). Für Octatrienal stand zu Beginn dieser Arbeit die Aufklärung der Biosynthese noch aus. Man kann spekulieren, dass Octatrienal in Analogie zu Octadienal aus der in Diatomeen vorkommenden 16:4ω1 Fettsäure gebildet wird (Volkman *et al.* 1989, d'Ippolito *et al.* 2002b). Um die Mechanismen der Biosynthese von fettsäureabgeleiteten Aldehyden in Diatomeen

weitergehend zu verstehen, wurde die deuterierte 9,10-[$^2H_2$]-16:4ω1 Fettsäure synthetisiert (**Publikation 4**). Während *T. rotula* C20-Fettsäuren in flüchtige α,β,γ,δ-ungesättigte Aldehyde (*polyunsaturated aldehydes*, PUA) und in ein noch nicht identifiziertes zweites Fragment umwandelt, bilden *A. formosa* und *G. parvulum* die α,β,γ,δ ungesättigten Oxosäuren 12-Oxododeca-5*Z*,8*Z*,10*E*-triensäure[6] (12-ODTE) bzw. 9-Oxonona-5*Z*,7*E*-diensäure (9-ONDE) und einen Kohlenwasserstoff als zweites Fragment (**Abb. 6**) (Homberg *et al.* 1999, Pohnert 2000).

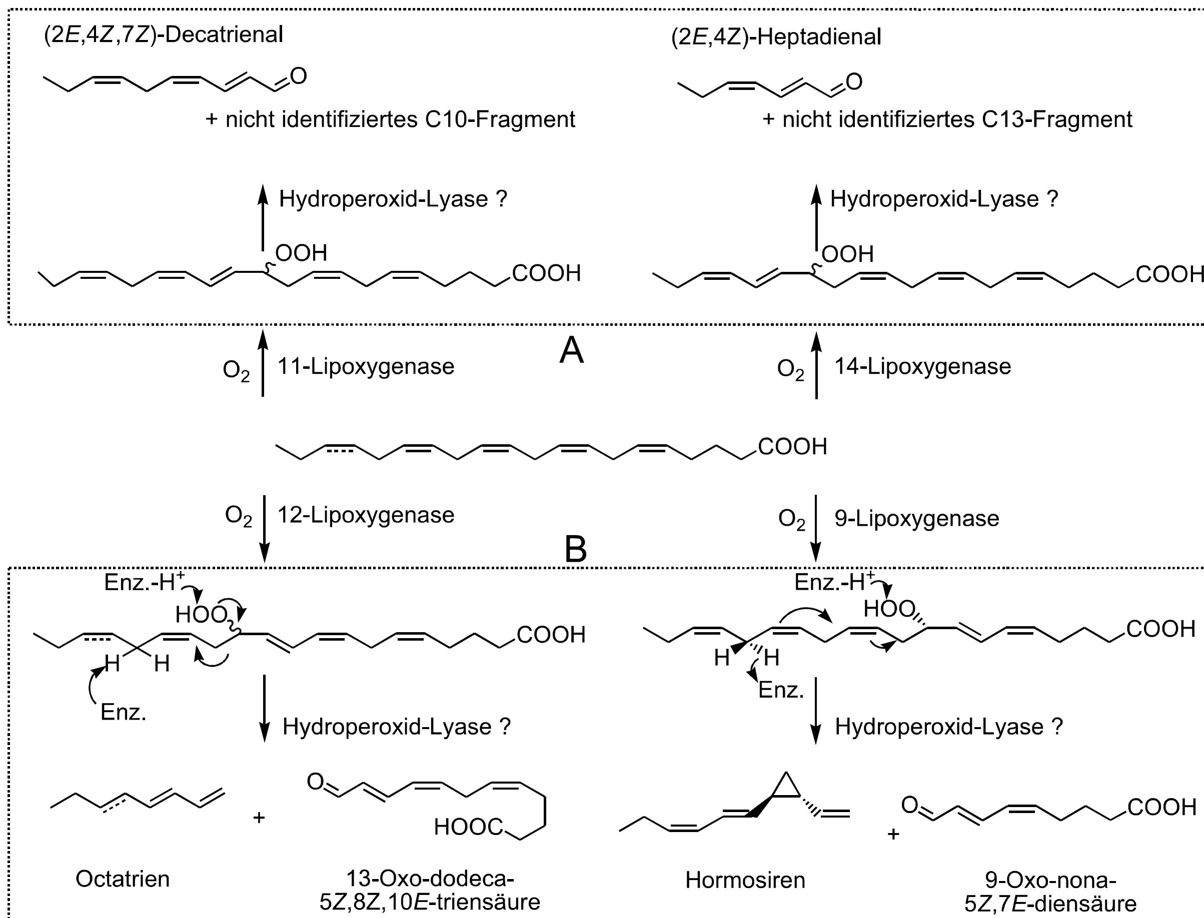

Abb. 6: Biosynthese α,β,γ,δ-ungesättigter Aldehyde aus Polyenfettsäuren durch Diatomeen. Eicosaepentaensäure bzw. Arachidonsäure werden durch vielfältige Lipoxygenase- und Hydroperoxid-Lyase Aktivitäten transformiert. *T. rotula* generiert Decatrienal/Decadienal bzw. Heptedienal und ein zweites nicht identifiziertes Fragment (**A**). *A. formosa* und *G. parvulum* hingegen generieren ein α,β,γ,δ-ungesättigtes Aldehyd mit azidischem Terminus und einen Kohlenwasserstoff bei (**B**).

[6] Nach IUPAC ist die vollständige Benennung **(5Z,8*Z*,10*E*)-12-Oxododeca-5,8,10-triensäure** im folgenden abgekürzt durch **12-Oxododeca-5*Z*,8*Z*,10*E*-triensäure**.

1.8 Aktivierung der Biosynthese $\alpha,\beta,\gamma,\delta$,-ungesättigter Aldehyde

Da in einer Diatomeenzelle keine freien Polyenfettsäuren vorliegen (Pohnert 2002), stellt sich die Frage nach der Initiierung der Biosynthese. In höheren Pflanzen kann durch Zellverletzung die Kompartimentierung eines Enzym-Substrat-Systems aufgehoben werden. Als Beispiel dafür kann die Aktivierung der Hydrolyse von Glucosinolaten genannt werden (Newman *et al.* 1992). In Diatomeen wurde eine verwundungsaktivierte Phospholipase A_2 Aktivität gefunden, die gespeicherte Fettsäuren aus Phospholipiden freisetzt. Nachfolgend werden die Fettsäuren durch Lipoxygenasen und Hydroperoxid-Lyasen in reaktive Oxylipine gespalten (Pohnert 2002), so dass sich eine wundaktivierte Kaskade von drei Enzymaktivitäten ergibt. Phopholipaseinhibitoren unterbinden die Initiierung der Kaskade und damit die Bildung ungesättigter Aldehyde (Pohnert 2002). d´Ippolito und Mitarbeiter (2004) fanden darüber hinaus ein Galaktolipid-hydrolysierendes Enzym, dass nach Zellverletzung Polyenfettsäuren aus beiden SN-Positionen des Monogalactosyldiacylglycerols (= MGDG) freisetzt. Diese Mechanismen können zu hohen lokalen Konzentrationen der reaktiven Aldehyde während der Fraßaktivität von Kopepoden führen und damit eine effektive Strategie der aktivierten chemischen Verteidigung im verdünnten Lebensraum der planktonischen Algen darstellen (Pohnert 2000). Die Aktivierung der Toxinbildung durch Zellverwundung während der Nahrungsaufnahme oder Verdauung im Darm eines Kopepoden ist eine interessante ökophysiologische Hypothese, die noch der experimentellen Bestätigung bedarf. Mit kommerziell erhältlichen fluoreszensmarkierten Phospholipiden (**Abb. 7**), die als Marker der Phospholipaseaktivität dienen (Hendrickson *et al.* 1999), lässt sich die Aktivierung der enzymatischen Kaskade im Kopepodendarm *in vivo* mit Hilfe konfokaler Mikroskopie nachweisen **(Publikation 14)**.

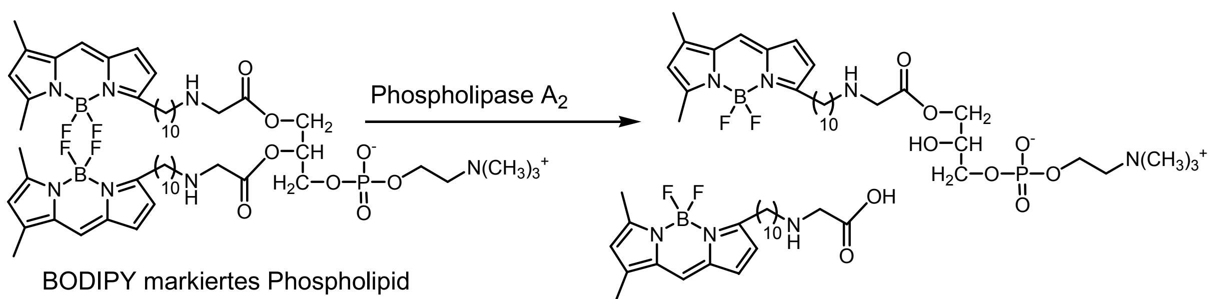

Abb. 7: Nachweis der Phospholipase-Aktivität durch ein (*Bis*-BODIPY FL *C11-PC*) markiertes Phospholipid. Nach Hydrolyse des Esters an der SN2-Position kann mit einer Anregungswellenlänge von 488 nm die emittierte Fluoreszenz detektiert werden.

1.9 Lipoxygenase vermittelte Transformation von Fettsäuren

Lipoxygenasen (LOX), definiert als Lineolat:Sauerstoff Oxidoreduktasen (EC 1.13.11.12), wurden bisher in Vertebraten, Algen, Pilzen und höheren Pflanzen nachgewiesen. Lipoxygenasen gehören zu der Familie der Dioxygenasen und enthalten ein über Aminosäureseitenketten gebundenes Eisenatom. Die Polyenfettsäure mit einem (1*Z*,4*Z*)-Pentadiensystem wird durch Protonabspaltung einer schwachen bisallylischen C-H-Bindung aktiviert (McGinley & van der Donk 2003). Dadurch entsteht ein delokalisiertes Pentadienyl-Radikal mit mehreren Reaktionszentren, das unter Bildung von (1*Z*,3*E*)-Hydroperoxyfettsäuren regiospezifisch mit molekularem Sauerstoff reagiert (Vliegenthart & Veldink 1977, Gardner 1991, Blée 1998a, Feussner & Wasternack 1998, Grechkin 1998, Feussner & Wasternack 2002) (**Abb. 8**).

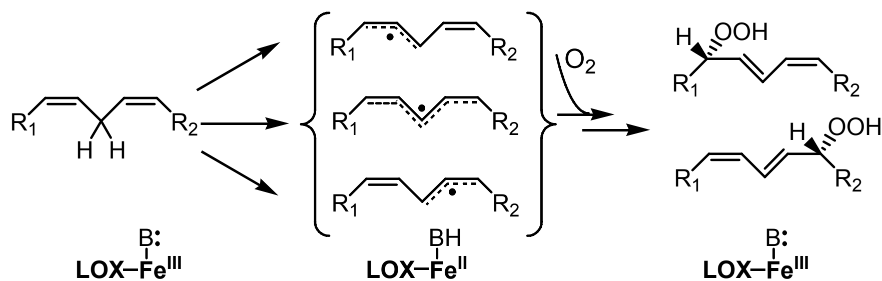

Abb. 8: Die Lipoxygenasereaktion: Nach Abstraktion des bisallylischen Protons entsteht ein delokalisiertes Radikal mit mehreren Reaktionszentren für die Addition von molekularem Sauerstoff (LOX-Fe^{III}: aktives Enzym, LOX-Fe^{II}: reduziertes inaktives Enzym; B: Base; nach McGinley & van der Donk 2003).

Je nach Regioselektivität der Lipoxygenasen wird bei den Blütenpflanzen zwischen Enzymen unterschieden, die den molekularen Sauerstoff in der Position 9 (9-LOX) oder in der Position 13 (13-LOX) der Linolsäure oder der α-Linolensäure einführen (Vick & Zimmerman 1983). Interessanterweise ist der Austausch einer Aminosäure im reaktiven Zentrum einer Lipoxygenase aus der Sojabohne hinreichend, um eine 13-LOX in eine 9-LOX zu transformieren (Hornung *et al.* 1999). Die Regioselektivität der Oxidation dieser Transformation wird durch die Geometrie des Aktivitätszentrums erreicht (Feussner & Kühn 2000). Diese Experimente deuten eine potenziell hohe katalytische Plastizität der Lipoxygenasen und mögliche Veränderungen enzymatischer Aktivitäten in der Abstammungsgeschichte der Pflanzen an.

In höheren Pflanzen werden die von Lipoxygenasen synthetisierten (9*S*)- oder (13*S*)-Hydroperoxide der Linolsäure und der α-Linolensäure durch eine Vielzahl, zum teil aufeinander folgender Enzymreaktionen zu oxygenierten Substanzen (= Phytooxylipine) umgesetzt (Blée 1998b, 2002, Feussner & Wasternack 2002). Die von der α-Linolensäure abgeleiteten Metaboliten werden auch als Octadecanoide bezeichnet, zu denen als wichtiger Vertreter die Jasmonsäure gehört (**Abb. 9**) (Creelman & Mullet 1997, Weiler 1997).

Octadecanoide sind an der Regulation zahlreicher physiologischer Prozesse beteiligt, wie den Verteidigungsmechanismen gegen Herbivorie und der Emittierung flüchtiger Substanzen (Boland *et al.* 1998). Diese können wiederum eine Signalwirkung auf Nachbarpflanzen haben (Arimura *et al.* 2002) oder Prädatoren der Herbivore anlocken (Dicke *et al.* 2003). Des Weiteren reguliert Jasmonsäure als Signalmetabolit entwicklungs- und stressabhängig die Expression verschiedener Gene, so dass sich der Abwehrstatus der Pflanze ändert (Wasternack *et al.* 1998) (**Abb. 9**).

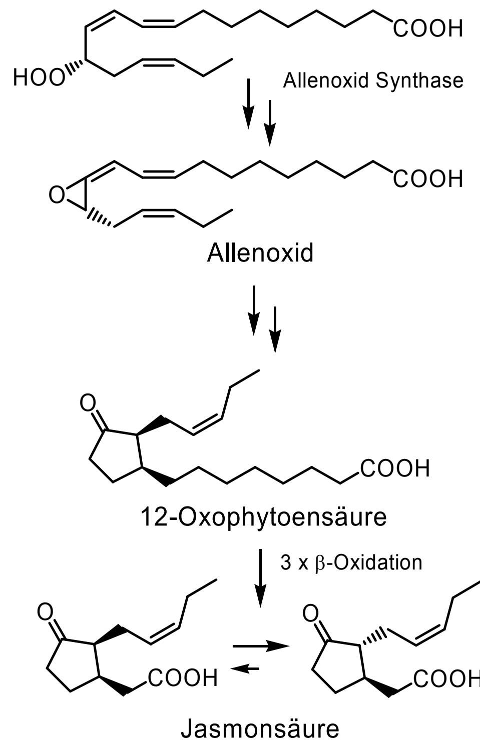

Abb. 9: Biosynthese der Jasmonsäure aus α-Linolensäure über den Allenoxid-Synthase-Reaktionsweg.

Dem intensiv erforschten Allenoxid-Synthase-Reaktionsweg stehen die weniger untersuchten anderen Zweige der Lipoxygenase vermittelten Reaktionswege gegenüber (Blée 2002). Über den Lipoxygenase/Hydroperoxid-Lyase Reaktionsweg bildet *Arabidopsis* aus α-Linolensäure stress- und wundinduziertes (2*E*)-Hexenal (Bate & Rothstein 1998). In den letzten Jahren nahm das Interesse an diesem Stoffwechselweg zu und wurde von Halitschke *et al.* (2003, 2004) hinsichtlich seiner Regulierung und ökologischen Relevanz bei der Tabakpflanze *Nicotiana attenuata* untersucht. Hydroperoxid-Lyasen wurden ursprünglich als „Aldehyd-Lyasen" in Bananenfrüchten beschrieben (Tressl & Drawert 1973), die für einen Bindungsbruch der Hydroperoxide von Fettsäuren verantwortlich gemacht wurden, bevor sie von Vick *et al.* (1976) in den Keimlingen der Wassermelone gefunden und als Hydroperoxid-Lyase (HPL) bezeichnet wurden.

Der durch eine Hydroperoxid-Lyase katalysierte C-C-Bindungsbruch wurde detailliert am Beispiel der (13*S*)-Hydroperoxy-9*Z*,11*E*,15*Z*-octadecatriensäure (13-HpOTE) und der (13*S*)-Hydroperoxy-9*Z*,11*E*-octadecadiensäure (13-HpODE) an verschiedenen höheren Pflanzen untersucht (Hatanaka *et al.* 1986, 1987, Noordermeer *et al.* 2001, Grechkin & Hamberg 2004). Die Hydroperoxid-Lyasen akzeptieren dabei ausschließlich das (13*S*)-Isomer der Hydroperoxylinolensäure als Substrat (Matoba *et al.* 1985).

Basierend auf Sequenzhomologien und der Elektronenspin-Resonanzspektrometrie wurde dieses Enzym als ein Cytochrom P450 Protein identifiziert und in die neue Gruppe CYP74B eingeordnet (Matsui *et al.* 1996). Cytochrome P450 Enzyme sind Häm-haltige Monooxygenasen, die im aktiven Zentrum hoch konservierte Cysteinreste aufweisen, die vermutlich die prosthetische Gruppe Protoporphyrin IX (hem b) binden. HPLn haben nur eine geringe Affinität zu Kohlenmonoxid und damit keine oder nur eine schwache Absorption bei 450 nm. Genau wie die Allen-Oxid-Synthasen brauchen die HPLn keinen molekularen Sauerstoff für ihre Aktivität und bilden daher eine eigene Gruppe (CYP74) innerhalb der Cytochrom P450 Enzyme.

Bis heute wird der katalytische Mechanismus kontrovers diskutiert. Nordermeer *et al*. (2001) favorisieren die Bildung eines Alkoxyradikals durch eine initiierende homolytische Spaltung der O-O Bindung des Hydroperoxids sowie die nachfolgende heterolytische Spaltung des gebildeten allylischen Ether-Kations. Greschkin *et al.* (2004) dagegen haben mit Hilfe von Silylierungsreagenzien das Hemiacetal 12-(1´-Hydroxy-3*Z*´hexenyloxyl)-9,11-dodecadiensäure abgefangen und als das eigentliche Produkt der HPL identifiziert. Die durch die HPL katalysierte Umlagerung des Hydroperoxids in das Hemiacetal erfolgt hierbei homolytisch. Das (3*Z*)-Hexenals und das Enol der Traumatinsäure werden dann über eine spontan ablaufende Spaltung gebildet (**Abb. 10**).

Ob HPLn membrangebunden oder in den Chloroplasten lokalisiert sind, ist unbekannt. Daher sind immunocytologische Methoden (Noordermeer *et al.* 2001) oder die Generierung von Fusionsproteinen mit YFP (*Yellow Fluorescent Protein*) notwendig, um den Wirkungsort der HPLn näher bestimmen zu können (**Publikation 10**).

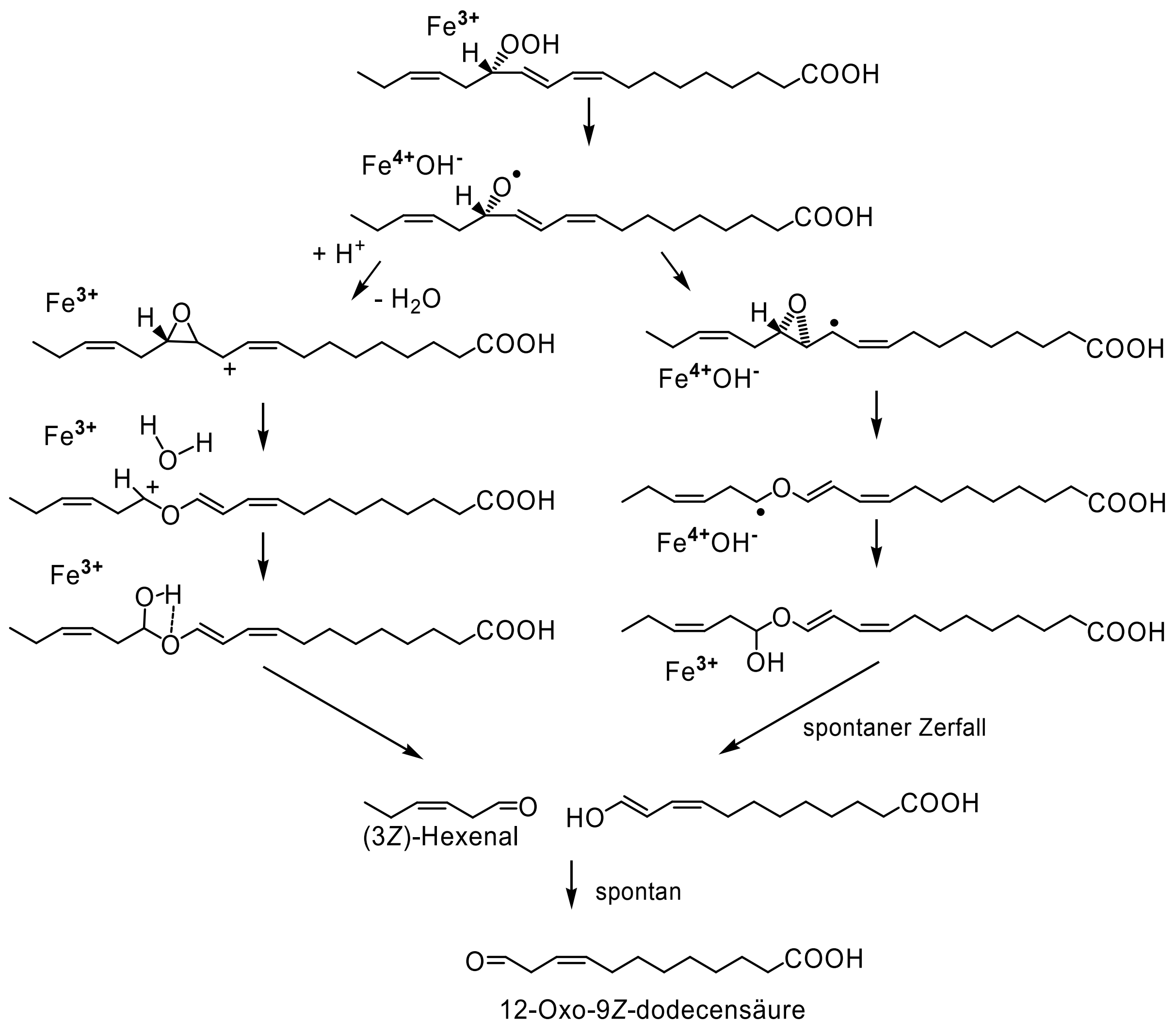

Abb. 10: Vorgeschlagene Mechanismen einer Hydroperoxid-Lyase. Die Bildung eines Alkoxyradikals unter Berücksichtigung der Chemie von Cytochrom P450 Enzymen geht der heterolytischen Spaltung nach Bildung eines allylischen Ether-Carbokations (Hatanka 1987) voraus (nach Noordermeer 2001). Das Hemiacetal ist das eigentliche Produkt der „Hydroperoxid-Lyase" und zerfällt spontan in den Aldehyd und das Enol der Traumatinsäure (Grechkin & Hamberg 2004).

In den letzten zwei Jahrzenten haben sich die Beobachtungen gemehrt, dass sowohl tierische als auch pflanzliche Lipoxygenasen weitere Aktivitäten besitzen. Zum einen wurde in der Koralle *Plexaura homomalla* ein Fusionsprotein aus einer Lipoxygenase- und einer Katalase-Aktivität gefunden, das Arachidonsäure über ein (8*R*)-Hydroperoxid in ein Allenoxid transformiert (Koljak *et al.* 1997). Zum anderen fanden Glasgow *et al.* (1986), dass eine partiell aufgereinigte 12-LOX aus Schweineleber-Leukozyten aus Arachidonsäure die 12-Oxosäure 12-Oxo-dodeca-5*Z*,8*Z*,10*E*-triensäure (12-ODTE) bildet. Während die Aktivität des Fusionsproteins an den Allenoxid-Synthase-Reaktionsweg erinnert und somit Parallelen zu der Biosynthese von Jasmonaten aufweist, scheint die Bildung der 12-ODTE durch eine konzertierte Lipoxygenase-Hydroperoxid-Lyase-Aktivität zu erfolgen. Salch und Mitarbeiter (1995) haben eine ähnliche Aktivität in einer aufgereinigten 13-LOX aus Keimlingen der

Sojabohne nachgewisen. Dieses Enzym setzt Linolensäure nicht nur ins korrespondierende 13-Hydroperoxid sondern auch in 1-Penten-3-ol und 13-Oxotrideca-9*Z*,11*E*-diensäure (13-OTA) um. Gleiches wurde in den Kotyledonen der Sojabohne gefunden (Kondo *et al.* 1995). Eine Verunreinigung durch eine Hydroperoxid-Lyase (HPL) konnte allerdings in allen Fällen nicht ausgeschlossen werden, da mit partiell aufgereinigten Enzymen gearbeitet worden ist.
Im Rahmen dieser Arbeit wurden die bisher sehr wenig untersuchten Lipoxygenase/ Hydroperoxid-Lyase Reaktionswege der niederen Pflanzen an zwei Beispielen, der Diatomee *Stephanopyxis turris* und dem Laubmoos *Physcomitrella patens*, untersucht. (**Publikation 9, 10, 13**). Dass Aldehyde zum einen die für den Verteidigungsmetabolismus höherer Pflanzen notwendige Expression von Genen aktivieren (Bate & Rothstein 1998) und zum anderen antimikrobielle (Croft *et al.* 1993) oder fungizide (Vaughn & Gardner 1993) Eigenschaften haben können, weist auf die Bedeutung der Hydroperoxid-Lyasen innerhalb der von Lipoxygenasen vermittelten Biosynthesen hin.
Bemerkenswert ist, dass Moose relativ selten von Tieren gefressen werden und meist frei von Schadorganismen sind (Zinsmeister *et al.* 1994). Nach bisheriger Kenntnis stellen sie die einzige virenfreie Pflanzengruppe dar (Zinsmeister *et al.* 1994). Diese Resistenz gegenüber Herbivorie und Parasitismus motivierte zahlreiche Forschergruppen (Zinsmeister *et al.* 1991) die Phytochemie der Laub- und Lebermoose hinsichtlich phenolischer, flavonoider und terpenoider Verbindungen zu untersuchen (Asakawa 1982). Über fettsäure-abgeleitete Metaboliten ist dagegen bis heute wenig bekannt, obgleich die vielfältigen hochungesättigten Fettsäuren der Moose eine hohe Produktdiversität an Oxylipinen erwarten lassen. Das Laubmoos *Physcomitrella patens* hat sich dabei zu einem interessanten Modelorganismus entwickelt (Schaefer 2002), weil es bis jetzt die einzige Landpflanze ist, die transformierte DNS (Schaefer *et al.* 1991) via *gene targeting* (Schaefer & Zryd 1997) durch spezifische homologe Rekombination mit hoher Einbaurate in ihr Genom integriert (Reski 1998, Reski *et al.* 1998). Dadurch können präzise spezifische Gene ausgeschaltet (*Knockout-Mutanten*) (Schaefer 2001) werden, deren biologische Funktion aufgrund der Haploidizität des Gametophyten[7] unmittelbar demonstriert werden kann (Girke *et al.* 1998, Strepp *et al.* 1998, Girod *et al.* 1999, Zank *et al.* 2002).
Knockout-Mutanten sind notwendig, um die Bedeutung einzelner Biosynthesen oder Metabolite für die Resistenz der Moose gegenüber Schädlingsbefall zu verstehen.

[7]Bei den Laubmoosen ist die grüne Pflanze der haploide Gametophyt, die Sporenkapsel der diploide Sporophyt, der mit dem Gametophyten verbunden ist.

Dieser Ansatz ist umso interessanter, da bekannt ist, dass die Deaktivierung von Hydroperoxid-Lyasen in transgenen Kartoffelpflanzen zu einem Anstieg des Schädlingbefalls durch Blattläuse führt (Vancanneyt *et al.* 2001). Im Rahmen der vorliegenden Arbeit wurde die Umsetzung von Polyenfettsäuren in Alkohole und Aldehyde bei *P. patens* untersucht (**Publikation 8**). Zwei an der Biosynthese der Oxylipine beteiligte Enzyme wurden kloniert und ihr Substrat- und Produktspektrum bestimmt (**Publikation 9, 10**). Mit Hilfe von Knockout-Mutanten wurde darüber hinaus die Regulation der Biosynthese untersucht (**Publikation 10**).

2. Übersicht zu den Publikationen

2.1 Fragestellungen der Publikationen

Insbesondere wurden folgende Fragestellungen in den Publikationen bearbeitet:

Lassen sich α,β,γ,δ-ungesättigte Aldehyde in Freilandversuchen im Phytoplankton nachweisen?

Um die Bedeutung von α,β,γ,δ-ungesättigten Aldehyden für die Interaktion von Phytoplankton und Zooplankton in aquatischen Systemen bestimmen zu können, ist eine robuste chemisch- analytische *in situ* Methode notwendig, die sensitiv und für Reihenuntersuchungen geeignet ist.

Welchen Einfluss haben α,β,γ,δ-ungesättigte Aldehyde auf den Reproduktionserfolg von *Calanus helgolandicus* im Jahreszyklus des Phytoplanktons in den Küstengewässern vor Roscoff?

An der marinen biologischen Station in Roscoff (Bretagne, Frankreich) sollten in Zusammenarbeit mit Dr. Serge A. Poulet und seinem Team neben der Bestimmung klassischer Parameter der Phytoplanktonsukzession (Spezieszusammensetzung, Chlorophyll *a* (= Chl *a*), Temperatur, POC und PON[8], usw.) insbesondere organisch-chemische Parameter des Phytoplanktons (α,β,γ,δ-ungesättigte Aldehyde und Polyenfettsäuren) bestimmt werden, um ihren Einfluss auf den Reproduktionserfolg der Kopepoden *Calanus helgolandicus* zu bestimmen. Der Reproduktionserfolg sollte anhand der Zahl gelegter Eier, der Schlupfrate sowie der Larvenentwicklung ermittelt werden.

Wie verbreitet ist die wundaktivierte Produktion von α,β,γ,δ-ungesättigten Aldehyden in der Klasse der Bacillariophyceae?

Um einen Überblick über das Potenzial der chemischen Verteidigung durch α,β,γ,δ-ungesättigte Aldehyde zu erhalten, wurden Reihenuntersuchungen von Diatomeen aus Algensammlungen und von frisch isolierten Spezies durchgeführt. Darüber hinaus sollte überprüft werden, ob sich Diatomeen hinsichtlich ihres durch Oxylipinspektrums in verschiedene Kategorien gruppieren lassen.

Ist die postulierte aktivierte Biosynthese der α,β,γ,δ-ungesättigten Aldehyde von Diatomeen auch im Kopepodendarm aktiv?

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[8]**POC**: partikulärer, organischer Kohlenstoff; **PON**: partikulärer, organischer Stickstoff

Es sollte getestet werden, ob nach Imprägnierung von Diatomeenzellen mit einem fluoreszent markierten Phospholipid die Phospholipase-Aktivität im Kopepodendarm mit Hilfe der konfokalen Mikroskopie beobachtet werden kann.

Haben Lipoxygenasen der niederen Pflanzen neben ihrer Aktivität als Dioxygenase weitere katalytische Funktionen?

Mit Hilfe des Modellorganismus *Physcomitrella patens*, wurden Lipoxygenase vermittelte Stoffwechselwege exemplarisch in niederen Pflanzen untersucht. Vor dem Hintergrund, dass Laubmoose genau wie Diatomeen reich an C20-Polyenfettsäuren sind, soll die katalytische Diversität von Lipoxygenase-Enzymen und das Oxylipinprofil der Lipoxygenase-vermittelten Biosynthesewege untersucht werden.

2.2 Inhaltsangaben

Publikation 1:

Colloquium on diatom-copepod interactions.

Paffenhöfer, G. A., Ianora, A., Miralto, A., Turner, J. T., Kleppel, G. S., d'Alcala, M. R., Casotti, R., Caldwell, G. S., Pohnert, G., Fontana, A., Müller-Navarra, D., Jonasdottir, S., Armbrust, V., Bamstedt, U., Ban, S., Bentley, M. G., Boersma, M., Bundy, M., Buttino, I., Calbet, A., Carlotti, F., Carotenuto, Y., d'Ippolito, G., Frost, B., Guisande, C., Lampert, W., Lee, R. F., Mazza, S., Mazzocchi, M. G., Nejstgaard, J. C., Poulet, S. A., Romano, G., Smetacek, V., Uye, S., Wakeham, S., Watson, S. & **Wichard, T.**

Marine Ecology-Progress Series 286, 293-305 (2005).

Das Kolloquium über die „Diatomeen/Kopepoden Interaktion“, das an der marinen Station „Anton Dorn“ in Neapel im Jahr 2002 stattgefunden hat, ist der Ausgangspunkt der vorliegenden Arbeit. Planktologen, Ozeanographen, Ökophysiologen und Chemiker diskutierten Fragen und Methoden zur Klärung der vielfältigen Interaktionen zwischen Diatomeen und Kopepoden. Ein Schwerpunkt bildete die Einbeziehung chemisch analytischer Ansätze, um eine auf Infochemikalien basierende Interaktion untersuchen zu können.

Ich habe während des Kolloquiums die Analytik von Diatomeenkulturen und Phytoplanktonproben angeboten sowie die Entwicklung einer einfachen Methode zur Quantifizierung ungesättigter Aldehyde in Phytoplanktonproben in Aussicht gestellt.

Publikation 2:

Determination and quantification of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes as pentafluorobenzyl-oxime derivates in diatom cultures and natural phytoplankton populations: application in marine field studies.

Wichard, T., Poulet, S. A. & Pohnert, G.

Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 814, 155-161 (2005).

Die Reduktion des Fortpflanzungserfolgs wird auf die Produktion von $\alpha,\beta,\gamma,\delta$-ungesättigten Aldehyden durch Diatomeen zurückgeführt. Zu Beginn dieser Arbeit wurden diese Metaboliten ausschließlich in Laborexperimenten nachgewiesen. Um in Freilandversuchen den Nachweis dieser Metabolite führen zu können, habe ich eine empfindliche *in situ* Derivatisierungsmethode entwickelt, die die reaktiven Aldehyde stabilisiert und in einem Standardlabor einer marinen biologischen Stationen angewendet werden kann. Ich war verantwortlich für die Planung, Realisierung und Auswertung aller Experimente, die notwendig waren, diese Methode zu entwickeln.

Publikation 3:

Survey of the chemical defence potential of diatoms: Screening of fifty one species for $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes.

Wichard, T., Poulet, S. A., Halsband-Lenk, C., Albaina, A., Harris, R., Liu, D. Y. & Pohnert, G.

Journal of Chemical Ecology 31, 949-958 (2005).

Um einen Überblick über die Verbreitung der wundaktivierten Produktion der $\alpha,\beta,\gamma,\delta$-ungesättigten Aldehyde innerhalb der Bacillariophyceae zu erhalten und Fütterungsexperimente mit definierten Mengen an ungesättigten Aldehyden in Laborversuchen planen zu können, habe ich das „toxische Potenzial" von 51 Diatomeenarten untersucht und ihre Aldehydproduktion quantifiziert. Die Kultivierung der Diatomeen, die Analysen sowie die Auswertung wurden von mir durchgeführt. Ich habe das Konzept ausgearbeitet und die erste Version des Manuskript geschrieben, das dann von Prof. Georg Pohnert und mir verbessert wurde.

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Colloquium on diatom–copepod interactions

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ABSTRACT: From 3 to 6 November 2002, a colloquium was convened at the Benthos Laboratory of the Stazione Zoologica Anton Dohrn on Ischia, Italy, with the goal of evaluating the present status of the effects of diatoms on their main consumers, planktonic copepods, and to develop future research strategies to enhance our understanding of such interactions. These included (1) toxic effects of diatom metabolites on copepods, particularly reproduction, and (2) nutritional effects of diatoms on juvenile to adult copepods. Key issues involved in the impact of diatoms on the dynamics of natural plankton communities *in situ* were also addressed. During the plenary session, the most recent advances on this topic were presented. The plenary session was followed by 3 working groups on (1) production of aldehydes by phytoplankton, (2) toxic and nutritional effects of diatoms on zooplankton, and (3) the chemistry of diatom defense, as well as of their nutritional quality. These working groups focused on suggesting future research needs for the different topics. As a result, several recommendations were outlined, including experimental studies. It became evident that interdisciplinary efforts are needed, involving chemists, oceanographers and experimentalists, since many of the biological observations under controlled conditions and *in situ* require an integrated approach, including chemical causation. Extensive field observations based on common protocols are also recommended for investigation of the intrinsic variability of such effects and their environmental controls. Laboratory experiments are seen to be essential for the full understanding of environmentally occurring processes.

KEY WORDS: Diatoms · Copepods · Toxicity · Nutrition

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INTRODUCTION

Main contributors: J. T. Turner and G. S. Kleppel

Phytoplankton blooms have been a major issue in marine ecology for more than a century (Mills 1989 and references therein). Diatoms are often major components of these blooms, and have long been considered as principal components of planktonic copepod diets (Dakin 1908, Marshall 1924, 1973, Clarke 1939, Mauchline 1998), enhancing copepod fecundity and thereby supporting major fisheries (Ryther 1969, Greve & Parsons 1977, Cushing 1989).

The diatom–copepod link was fully accepted in biological and fishery oceanography by the mid-twentieth century. However, substantial data refuting the diatom → copepod → fish model began to emerge in the late 1980's and continued throughout the 1990's. Increased appreciation for the 'microbial loop' (Azam et al. 1983) and the importance of heterotrophic protists in grazing the dominant picoplanktonic phytoplankton somewhat diminished the importance of copepods as grazers of diatoms. Substantial, or even preferential, predation of copepods on heterotrophic protists and dinoflagellates (Sherr et al. 1986, Stoecker & Capuzzo 1990) seemed to reduce the importance of diatoms as copepod food. In fact, some copepods previously regarded as 'herbivores' have been shown to readily ingest heterotrophic protists and sustain fecundity on these food taxa (Ohman & Runge 1994), and such copepod production can contribute to larval fish recruitment during periods of low phytoplankton chlorophyll (Runge & de Lafontaine 1996). Additionally, copepod egg production was observed to be more closely correlated with heterotrophic protist and dinoflagellate biomass than with that of diatoms (Kleppel et al. 1991, Ohman & Runge 1994). Thus, by the 1990's, it was clear that the diatom–copepod trophic linkage was only part of a complicated planktonic food web, rather than a predominant food chain (reviewed by Kleppel 1993, Turner & Roff 1993, Verity & Paffenhöfer 1996).

In addition to the aforementioned studies, other information began to accumulate in the 1990's that questioned the beneficial role of diatoms as copepod food. Briefly, a body of evidence (reviewed by Paffenhöfer 2002, Ianora et al. 2003) indicated that ingestion of some diatom species by females of some calanoid species could decrease their reproductive success due to the presence of teratogenic, or reproduction-inhibitory chemicals in these diets. Unsaturated aldehydes (the term 'aldehyde(s)' is used in the text as an abbreviation to cover biologically active $\alpha,\beta,\gamma,\delta$- or α,β-unsaturated aldehydes) in some diatom species inhibited mitosis in developing copepod eggs and sea urchin embryos (Miralto et al. 1999), and induced abnormal development of copepod nauplii in both the laboratory (Poulet et al. 1995, Carotenuto et al. 2002) and in the sea during periods of diatom abundance (Laabir et al. 1995a, 1998, Miralto et al. 2003, Ianora et al. 2004). Diets consisting of several species of diatoms have been shown to reduce egg viability in several species of copepods (Ianora et al. 1995, 1996, Ban et al. 1997, Lee et al. 1999, Starr et al. 1999, Lacoste et al. 2001). The reduction of egg hatching success is proportional to the length of exposure of egg-producing females to diatom diets (Laabir et al. 1995b, Turner et al. 2001), and to diatom concentrations (Chaudron et al. 1996).

As an alternative, some authors have argued that poor copepod egg production and egg viability result from nutritional inadequacies rather than teratogens in diatom diets of egg-producing female copepods (Jónasdóttir et al. 1998). Numerous correlations have been found between copepod fecundity and various elemental and biochemical components of maternal diets such as carbon, nitrogen, protein, carbohydrate, amino acid, and fatty acid content (Jónasdóttir 1994, Pond et al. 1996, Kleppel et al. 1998a,b, Koski et al. 1998). Schmidt & Jónasdóttir (1997) showed that copepod egg production increased when additional taxa were added to unialgal diets of nutritionally poor cyanobacteria. Copepod egg-hatching success was correlated to size and biochemical content (protein, carbohydrate, lipid) of eggs, and these parameters were correlated to biochemical compounds in natural food assemblages (Guisande & Harris 1995). Both copepod egg production and egg hatching have been correlated to dietary levels of fatty acids or amino acids in laboratory-cultured (Jónasdóttir & Kiørboe 1996, Guisande et al. 2000) and natural (Pond et al. 1996) diets. Other studies have revealed variable rates of copepod egg production on diets of natural phytoplankton dominated by various taxa (Tang et al. 1998, Irigoien et al. 2000a), with high variability in the effects on fecundity when diatoms were dominant (Irigoien et al. 2000b).

To consider the controversial issue regarding the relationship between diatoms and copepods, a meeting was convened at the Stazione Zoologica Anton Dohrn Benthos Laboratory, Ischia, Italy, from 3 to 6 November 2002. The goal of this meeting was to evaluate the present status of diatom–copepod interaction research, including unpublished results, and consider means by which to explore the effects of nutritional quality and the influence of secondary metabolites on the relationships between diatoms and copepods. Thirty-seven scientists from 10 countries participated. A consensus emerged from the meeting that a research agenda ought to be developed, aiming towards a comprehensive understanding of one of

the most fundamental and long-recognized interactions in the sea, which is that between diatoms and copepods.

In the following sections we will first present reports from 3 working groups: The first group, concentrating on phytoplankton, largely focused on aldehyde production and the mechanisms leading to such production. The second, concentrating on zooplankton, developed questions about how diatoms affect copepod reproduction and other population dynamics variables. The third group, concentrating on chemistry, had 2 points of interest, i.e. the chemistry of diatom defense and the nutritional chemistry of diatoms. In the last section we present considerations for future research, followed by concluding remarks.

PRODUCTION OF ALDEHYDES BY PHYTOPLANKTON

Main contributors: M. Ribera d'Alcalà and R. Casotti

This section originates from a working group evaluating aldehyde production, and possible mechanisms initiating or modulating the formation of aldehydes from phytoplankton. The production of defense molecules against predation are widely known for both terrestrial (Rosenthal & Berenbaum 1991) and marine (McClintock & Baker 2001) higher plants. Much less is known about the production of these molecules in marine phytoplankton. Unsaturated aldehyde production has been shown to vary among different phytoplankton species, different genotypes of the same morphological species and different sites. Also, diatom aldehydes have been shown to differ in their toxicity, and those with an α,β-unsaturation and longer chain length are more toxic to animals than others (Adolph et al. 2003, Romano et al. 2003). However, it is not yet clear which phytoplankton populations produce aldehydes in nature, because relatively few species have been biochemically analyzed to date. Many of these are, indeed, species that form blooms, which reinforces the idea of a positive feedback between the production of aldehydes and increased diatom cell numbers at sea. On the other hand, species that do not form blooms have also been shown to produce the same or similar aldehydes, suggesting that chemical defense, per se, is not a sufficient requisite for increased population growth. Since all aldehydes require multi-step enzymatic catalysis, important questions arise about the genetic control of such processes. Such questions can be addressed in the near future using molecular tools, made possible by the recent sequencing and annotation of the marine diatom *Thalassiosira pseudonana* genome (Armbrust et al. 2004).

If the production of unsaturated aldehydes depends on a cell-controlled activation mechanism, their release should depend on a trigger. This trigger could be external (wound induction, presence of grazers, nutrient concentration, nutritional differences) or endogenous (growth stage, biochemical composition). Once chemical analytical methods advance, studies of the physiological control of aldehyde production at the cellular scale should be initiated, in order to dissect the endogenous mechanisms of aldehyde production by diatoms. Toxin production in phytoplankton is often related to different phases of the growth cycle (Imada et al. 1991, Schmidt & Hansen 2001), or even to cell-cycle phases (Bates et al. 1993, Bolch et al. 2002). It will have to be shown whether aldehyde production is higher in senescent cells or exponentially growing cells. The former would imply that grazers feeding at the end of a bloom are more affected than early-bloom grazers, while the latter would imply the opposite. Both scenarios would have strong implications for recruitment and trophic interactions at sea.

External factors triggering or modulating aldehyde production also need to be investigated, and light and nutrients are potentially important factors to consider. In freshwater chrysophytes producing the same aldehydes, physiological conditions, and light and nutrient availability, strongly modulate their production, which has strong implications for the phytoplankton community composition (Watson & Satchwill 2003). If similar results are obtained for marine bloom-forming species, this may represent a strong determinant for bloom dynamics. For example, the high-light conditions that diatoms experience when trapped in the surface layers of the water column as a consequence of stratification may have an effect on such reactive molecules. UV activation or modulation of toxicity has, in fact, also been observed for similar compounds (Wiegman et al. 2002).

If the production of such compounds is rewarded by a higher survival rate of the clones, the energy investment must be low; otherwise other phytoplankters would outcompete the producers due to reduced grazing pressure (Yoshida et al. 2003). Chemical defenses may also be effective against other competing autotrophs. For example, Casotti et al. (2001) suggested that diatom-derived aldehydes have an allelochemical function, since they arrest the proliferation of other diatom cells. If aldehyde production is related to the peak phase of a bloom, then aldehydes may be a control mechanism to prolong algal survival when nutrients become limiting. Such an altruistic behavior may be mediated by active mechanisms at the cellular level, such as programmed cell death (i.e. apoptosis), as has been demonstrated in *in vitro* experiments on different animal and plant models (Casotti et al. 2001,

2005, Romano et al. 2003). The release of aldehydes into the seawater in quantities that inhibit growth of competitors appears to be unrealistic (Pohnert 2000), but a quantitative assessment of aldehyde production during bloom conditions is still lacking. On the other hand, unsaturated aldehydes are produced by other algal groups in freshwater environments, in such high quantities as to cause serious socio-economic problems (Watson 2003). Unsaturated aldehyde production appears to be regulated by algal physiology, and therefore a possible role of these compounds as semiochemicals (i.e. pheromones, kairomones and allelogens) in freshwater habitats has been advanced (Watson & Satchwill 2003). Also in the ocean, there is evidence for the presence of secondary microalgal metabolites with a signaling function (Wolfe et al. 1997), and the question is whether diatom-produced aldehydes may also have such a role.

Diatoms, as a group, are major contributors to carbon fixation in the ocean (Nelson et al. 1995), and a significant fraction of diatom biomass is effectively transferred through the marine food web. Pathways therefore exist which allow diatom grazers to safely grow, reproduce and transfer their carbon to their consumers. This may be based upon avoidance or limited intake of toxic species, or on resistance mechanisms of copepod species. Unicellular grazers might be unaffected by diatom-derived aldehydes, or could even neutralize their effects. Such grazers have long been considered to be more effective than copepods in consuming phytoplankton (e.g. Frost 1987), and a recent study supports that view (Calbet & Landry 2004).

A thorough analysis of overlooked defense mechanisms will improve our understanding of the functioning of food webs and, thereby, of carbon drawdown by the ocean and the possible changes due to community shifts in the context of global change. It will also allow a better understanding of the impact of the diatom–copepod interactions on marine resources (fishery) and on the life strategies of pelagic and benthic organisms (marine biodiversity).

TOXIC AND NUTRITIONAL EFFECTS OF DIATOMS ON ZOOPLANKTON

Main contributors: G. S. Caldwell and J. T. Turner

This section originates from a working group which mainly focused on formulating questions about the ways and means by which diatoms affect copepod reproduction, and other population dynamics variables. The general mechanisms considered were toxicity and nutritional inadequacy. Although some results point to the second issue, the information available on the nutritional value of diatoms *in situ* is still scarce. It was emphasized that copepod species and also diatom species differ in their physiology, and that we possess hardly any knowledge as to how the ingestion of different diatom species would affect different copepod species, with a few published exceptions. The evidence that we have of a copepod species' feeding behavior in diatom blooms (e.g. Fessenden & Cowles 1994) is limited, because such blooms and associated particle qualities can vary widely in their composition, leaving room for a range of behavioral responses of the different copepod species. Various aspects of the known effects of diatoms on copepod reproduction and nutrition are presented in earlier and following sections. Here we limit the presentation to general questions and ideas concerning diatom–copepod interaction research, covering field and experimental observations.

How often are diatom toxicity and/or nutritional inadequacy associated with diatom blooms? To answer this question the quantifications needed over time would have to include copepod ingestion rates of the different bloom particles, subsequent egg production and hatching, and naupliar and copepodid development and mortality (post-embryonic fitness). Also, a thorough analysis of phytoplankton composition and abundance, and the phytoplankton species' chemical composition (see 'Chemistry of diatom defense' and 'Nutritional chemistry of diatoms') is needed. Answering the above-mentioned question would require oceanographic observations, as well as specific laboratory experiments simulating field conditions as closely as possible. Observations of Ban et al. (2000) demonstrated clear negative effects of diatom blooms, as reflected by the asymmetry of nauplii which had hatched under the bloom conditions. The field studies ought to be designed to understand the ecological significance of toxic molecules for natural copepod populations. Again, it is essential to distinguish between the effects of toxicity and nutritional value.

Following this, are different species of calanoid copepods, which are the main copepod consumers of diatoms, with different feeding strategies similarly affected by the same diatom conditions? Here, the possibility of detoxification should be considered. While Miralto et al. (1999) speculated as to the apparent lack of detoxification systems in copepods, Ianora et al. (2003) showed that the same diatom species could induce different effects in different copepod species. Benthic invertebrates have revealed pronounced species-specific variability in the sensitivity of their embryos to aldehyde toxicity (Caldwell et al. 2002). No inhibitory effects on reproductive and population growth parameters were observed following ingestion of the benthic diatom *Nitzschia closterium* by the

harpacticoid copepod *Tisbe biminiensis* (Pinto et al. 2001). Nutritional quality should affect growth and survival of nauplii and copepodid stages, as has been shown for *Calanus helgolandicus/pacificus*, where the ingestion of different diatom food species resulted in differing mortality rates and generation times (Paffenhöfer 1970). Similar findings were obtained by Carotenuto et al. (2002). The results of Koski et al. (1999) also revealed that food quality affects growth and development of calanoid copepods.

What are the mechanisms of transfer and accumulation of aldehydes and other toxic components, and their fate in growing copepods, as well as in eggs? There could be delayed effects, as has been shown for polychaete larvae cultured at very low aldehyde concentrations (0.0001 to 0.01 $\mu g\ l^{-1}$). These had hatched normally, but expressed pronounced anatomical deformities when they metamorphosed to nechtochaete larvae (Lewis et al. 2004). This, again, could vary with copepod species, but also with their physiological state, as well as their particle environment.

It was pointed out that the methods of quantifying rates of ingestion, reproduction, mortality, etc., as well as egg viability, ought to be standardized; this would be essential for a sound comparison of results originating from different investigators working at different locations on different species.

CHEMISTRY OF DIATOM DEFENSE

Main contributors: G. Pohnert and A. Fontana

This section focuses on current and future strategies for the analyses of unsaturated aldehydes, the investigation of their biosynthetic origin and the regulation of their formation.

After the initial discovery of the inhibitory effect of unsaturated C10-aldehydes on the hatching success of copepods (Miralto et al. 1999), both mechanistic and methodological approaches were developed to gain an in-depth understanding of the 'defensive chemistry' of diatoms. During recent years, this has resulted in major progress towards the understanding of reactive aldehyde biosynthesis. Nevertheless, a detailed mechanistic and ecological understanding of the significance of diatom defense compared with the effects of overall food quality is yet to be achieved. Several different analytical approaches have been developed to detect unsaturated aldehydes in diatom cultures and in the natural environment (summarized in Table 1).

The data obtained to date from different diatom species and strains indicate that the formation of reactive aldehydes is not a universal property of all diatoms but is strongly variable among species and strains. For example, while *Thalassiosira rotula*, isolated from the Gulf of Naples, produces high amounts of 2*E*,4*Z*,7*Z*-

Table 1. Extraction and detection methods for reactive aldehydes from marine and freshwater diatoms. NMR: nuclear magnetic resonance

	Wittig-based derivatization[a,b]		Solid-phase microextraction[c,d]	HPLC-MS
	Metabolically unknown samples (single species and phytoplankton)	Metabolically known organisms		
Amount of wet weight required	2–5 g	800–900 mg	ca. 300 mg	ca. 300 mg
Information	Identification of unknown metabolites (assisted by NMR)	Identification of known metabolites by GC-MS	Identification of known compounds with synthetic standards using GC-MS but difficult to quantify	Identification of known compounds with synthetic standards
Time requirement	5 d	24 h	3 h	1 h
Sample preparation	Wounding/extraction/ derivatization/ chromatography	Wounding/extraction/ derivatization/ chromatography	Wounding/extraction/ chromatography	Wounding/ chromatography
Main advantages	Derivatized samples are stable; Field measurements possible; Unambiguous identification; No standards required		Fast and sensitive; Suitable for screenings of different cultures	Fast and sensitive; Reliable quantification; Identification of polar oxylipins

[a]After d'Ippolito et al. (2002a); [b]after d'Ippolito et al. (2002b); [c]after Pohnert (2000); [d]after Watson et al. (1999)

decatrienal and *2E,4E,7Z*-decatrienal and traces of *2E,4Z*-decadienal, *Skeletonema costatum* from the Adriatic Sea produces the volatile C8 aldehydes *2E,4Z*-octadienal and *2E,4Z,7*-octatrienal as major metabolites. Recent studies have shown that different aldehydes are derived from polyunsatured C20 and C16 fatty acids (Pohnert 2000, d'Ippolito et al. 2002a,b, Pohnert et al. 2002), and that these compounds are produced not only by laboratory cultures but also by field-collected diatom species (A. Fontana & G. Pohnert unpubl. data). Yet, seston from marine and freshwater habitats and cultured diatom species have been identified that do not produce any of these or related oxylipins (T. Wichard, A. Fontana & G. Pohnert unpubl. data). This finding highlights the need for further screening of marine diatoms for their capability to synthesize unsaturated aldehydes. The laboratories in Jena (pohnert@ice.mpg.de), Napoli (afontana@icmib.na.cnr.it; romano@szn.it) and Calgary (swatson@ucalgary.ca) will perform these analyses and will give advice on sample preparation upon request.

Methods have been developed that provide tools for the investigation of the aldehyde production of diatom cultures or field samples and that can be used for a comparative screening of some of the major bloom-forming diatoms. In this regard, it is of primary interest to analyze samples of phytoplankton collected during algal blooms under well-known marine environmental conditions. Assisted by parallel studies of the *in situ* dynamics of diatom and copepod populations, this work will clarify not only the presence of aldehydes in major marine algal blooms, but also the ecological role of these compounds under natural conditions. This will allow estimation of the distribution of the potentially inhibitory species in the natural environment. A recent comparative study of *Thalassiosira rotula* from the Gulf of Naples and coastal California showed that, despite being genetically closely related and morphologically nearly identical, the Naples isolate produced high amounts of antiproliferative aldehydes (same as above), while the California strain completely lacked the ability to form these compounds (Pohnert et al. 2002). These 2 strains could be used in further comparative studies for the evaluation of the effects of oxylipins compared to the overall effects of food quality.

A major task in the near future will be the development of chemical analytical methods that will allow us to investigate processes in the copepod gut and tissue, in order to evaluate the possible uptake and distribution of reactive aldehydes towards target molecules, cells and organs. Correlative studies have shown that the effect of externally added aldehydes is the same as that observed after ingestion of aldehyde-producing diatoms. However, the high concentrations of 1 to 2 µg ml^{-1} decadienal required to induce an effect, and the unresolved question of local targeting of the metabolites in the copepods, do not allow the conclusion that these metabolites act directly and exclusively as defensive molecules to be made. Chemical methods, as well as bioassays and labeled probes, will be required in the future to bring a physiological understanding to this discussion. By using whole animals, it should be possible to investigate the distribution and location of reactive aldehydes after feeding. New strategies for the administration of reactive aldehydes with food will have to be developed. Whether this can be done, e.g. with microcapsules containing radioactive stable isotope-labeled samples of diatoms, will have to be established by collaboration among ecologists and analytical chemists.

Initial biosynthetic studies suggest that the production of these metabolites is triggered by mechanical damage (Pohnert 2000). After wounding, the release of eicosanoic fatty acids by a phospholipase A_2 is the first step of an enzymatic cascade involving a lipoxygenase and hydroperoxide lyase (Pohnert 2002). Studies of different diatom genera indicate that the biosynthesis of unsaturated aldehydes follows a similar pathway involving the same metabolic precursors and enzymes (Pohnert & Boland 2002, d'Ippolito et al. 2003). However, different diatom species can produce unsaturated aldehydes with differing biological activities, as has been shown for *Skeletonema costatum* and *Thalassiosira rotula*, which produce C7, C8 and C10 aldehydes (d'Ippolito et al. 2002a,b).

Future work will lead to a more profound understanding of how these molecules are released into the environment during growth, senescence and the feeding process, and will give additional information on the cell targets of the molecules. The influence of environmental conditions on the ability of diatoms to form reactive metabolites will also be addressed. To date, no information is available as to whether external chemical signals, such as light, nutrients or temperature, affect the quantity of aldehydes produced per cell. Such information would lead to a better understanding of regional differences in the defensive potential of diatoms. The greatest challenge for future years will be the search for additional or alternative primary and secondary metabolites that can influence the reproductive success of copepods.

NUTRITIONAL CHEMISTRY OF DIATOMS

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This section focuses on the differentiation of toxic effects from nutritive ones, i.e. compounds essential for copepod growth and development, with some sugges-

tions for future research. The nutritional biology of marine copepods in general has recently been investigated by, e.g., Støttrup & Jensen (1990), Jónasdóttir (1994), Ederington et al. (1995), Kleppel & Burkart (1995), Pond et al. (1996), Guisande et al. (1999), and Klein Breteler et al. (1999). The focus of most of these studies was the effects of different algae on copepod performance. With regard to diatoms, monospecific diatom cultures have been shown to induce variable effects on copepod egg production and hatching (Jónasdóttir & Kiørboe 1996, Ban et al. 1997, Paffenhöfer 2002), ranging from being an excellent food source to being detrimental for copepods. The main issue is that in many studies it is not clear whether, or under which conditions, reduced egg production and hatching rates are due to toxins (e.g. certain aldehydes) in diatoms or to a nutritional inadequacy of a diatom diet.

From general knowledge of nutrition, it has been established that a whole variety of biochemicals and minerals must be supplied by the food. As a result, there is a high probability that a monospecific algal diet is nutritionally unbalanced or even deficient in 1 or more substances, and hence of inferior quality to a multispecies food mixture. Thus, it is easier for a monospecific algal culture to be nutritionally inadequate than the natural mixed diet copepods encounter in the field.

Nutritional chemistry studies of zooplankton in general have mainly focused on the fatty acid composition of the food (e.g. Müller-Navarra 1995, Jónasdóttir & Kiørboe 1996, von Elert 2002) and also, but to a lesser extent, on the amino acid composition (Kleppel & Burkart 1995, Guisande et al. 1999) and, recently, sterols (von Elert et al. 2003, Hassett 2004). Although considerable advances have been made in our knowledge of the nature of the biochemicals that might be important for limiting zooplankton growth and egg production during the last decade (Støttrup & Jensen 1990, Pond et al. 1996, Müller-Navarra et al. 2000, 2004, von Elert 2002, Becker & Boersma 2003), it is not yet clear what might be missing from the pure monospecific diatom cultures that seem to be of suboptimal food quality. Recent studies suggest that linolenic acid (18:3(n-3), von Elert 2002 for *Daphnia*) or cholesterol (Hassett 2004) may be underrepresented in certain diatoms. In contrast, although eicosapentaenoic acid, 20:5(n-3), is 1 important semi-essential nutrient for zooplankton growth, the concentrations in diatoms are generally very high, so these compounds are not expected to be limiting. However, evidence from aquaculture research indicates that easily degradable fatty acids like 20:5(n-3) have to be offered in a protected and bioavailable form, and that too high a concentration can have a detrimental effect on the consumer (McEnvoy et al. 1995, Coutteau et al. 1997, Jüttner 2001).

Separating potential toxic effects from nutritional inadequacy or deficiency is a major challenge in the nutritional biology of zooplankton. The effect of toxic and nutritionally deficient diets can both result in similar or identical phenomena (i.e. exhibit the same phenotypic features) in copepod reproduction, hence they cannot be easily distinguished. Separating the effects may at times be impossible if both alternative causes are not addressed in the measurements of factors in the field and in the design of laboratory studies. One of the simplest ways to differentiate between toxicity and nutritional deficiency is by means of an indirect test in which a deficient nutritional substance should have an enhancement effect when added as a supplement to a copepod culture, whereas a toxic substance should have a deteriorating (impairing) effect on the consumer when added. In addition, measurements of ingestion to evaluate selection are necessary. The substances in question have to be chemically characterized, added in a bioavailable form (e.g. as microcapsules), and in a way which does not lead to dilution of the food solution to be tested.

Additionally, the biochemicals that are potentially the most limiting resources need to be identified so that the above-mentioned experimental approach can be applied. Recent research has revealed that a balance of various nutritional components is important to ensure a high nutritional value of phytoplankton for zooplankton (Kleppel 1993, Anderson & Pond 2000). Even though, according to Liebig's law of the minimum, only 1 resource can be limiting at a time, a whole suite of essential nutrients should be required for somatic growth, egg production and hatching, because these 3 physiologically different production processes can have different nutritional requirements. In addition, because many nutritional substances can be stored in the copepod, experiments must ensure that these body reserves are depleted in the experimental animal before conclusions can be made about them being limiting under certain food conditions.

To fully understand the role of diatom quality in controlling copepod secondary production, only experiments and chemical analyses which integrate both toxic and nutritional aspects of food biochemistry will be successful in improving our understanding of the role of nutrition and toxicity in diatom–copepod interactions, and will resolve why some diatom species better support copepod population growth than others.

FINAL CONSIDERATIONS FOR FUTURE RESEARCH

Several suggestions for future research on diatom–copepod interactions have been presented in the previous sections. In addition, we want to complement

these suggestions with some general thoughts, including methods, to be followed by specific proposals for future field and experimental efforts investigating diatom toxicity to copepods and nutritional value of diatoms, taking into account various recent observations on the interaction between copepods and their food organisms.

General thoughts on methodology

To obtain insights into the relationships between individual diatom species and the reproduction of individual copepod species, proven methodologies should be applied, following Ianora et al. (1995, 1996). The methods used for comparative purposes, including quantifying rates of ingestion, reproduction, mortality, egg viability, and chemical analyses, should be standardized. The approach of Nejstgaard et al. (2001a,b) is recommended for obtaining information about the reproduction of individual copepod species feeding on natural food spectra. This could be complemented by the evolving methodology of Nejstgaard et al. (2003), which reveals the ingested food species via DNA markers in the gut contents. The latter methodology should also be used in conjunction with that of Ianora et al. (2004) when studying the extent to which juvenile stages are affected by ingested food taxa *in situ*. Stage-specific ingestion experiments should apply a single food taxon of proven 'good' quality as a control; focus should be on whether juvenile stages select for or against certain food types, and what biochemical compound may characterize such food types. A promising way to study these integrated effects on larval recruitment in natural plankton is by the use of mesocosms with minimally manipulated volumes (e.g. Nejstgaard et al. 2001b).

Diatom toxicity to copepods

Most *in situ* observations of deleterious effects were made when 1 or 2 species of diatoms dominated in coastal regions (Ianora & Poulet 1993, Miralto et al. 1999, 2003, Ianora et al. 2004). To gain additional knowledge of the *in situ* effects of major diatom blooms consisting of 1 or 2 species, it may be useful to study other coastal regions and shallow shelves known for such or similar events over time. The following regions could be promising: (1) Mississippi River plume, where Turner & Tester (1989) found that, at certain times, single diatom species such as *Skeletonema costatum* or *Thalassiosira* spp. comprised 79 to 85% of all cells; (2) the west coast of Scotland, where Marshall & Orr (1930) reported a nearly monospecific bloom of *S. costatum*, which provided food for emerging populations of *Calanus finmarchicus*; (3) Campeche Banks in the Gulf of Mexico, with major diatom blooms (Belousov et al. 1966); and (4) coastal waters off south and western South Africa, with frequent diatom blooms due to upwelling (e.g. Pitcher 1988). In addition, certain offshore regions, such as the Arabian Sea during the SW monsoon (e.g. Smith 2001), and other wind-driven upwelling areas, e.g. the NW coast of the Iberian Peninsula, are known for pronounced diatom blooms. Contrasting with diatom toxic effects, there are also studies showing that diatoms have no or limited negative effects on the egg viability of various calanoid copepod species (Pond et al. 1996, Irigoien et al. 2002), or have only small effects (Miralto et al. 2003, summer). These studies were carried out when the diatom assemblage consisted of several species and concentrations were lower than those observed in early spring by Miralto et al. (2003). Observations range from 'near-catastrophic' events of diatom toxicity to copepod reproduction to cases in which the effects were far less noticeable, if at all (e.g. Laabir et al. 1995a). Unfortunately, the authors of most previous studies did not quantify the particle types actually ingested by the copepods, and no study correlating aldehyde production with ingested food is known. As it is possible that toxic diatoms are not ingested, the claims of the absence of negative effects caused by diatoms lack the basic information needed to substantiate the conclusions. It should be further emphasized that future *in situ* studies of food particle effects on zooplankton need to quantify both the types and amounts of food ingested (see Turner et al. 2001). This was achieved in the following time-series study: A series of 30 to 40 d mesocosm studies on the Norwegian west coast (J. C. Nejstgaard unpubl. data) showed a large variation in net production of copepods and other zooplankton between seasons and years. The mesocosms dominated by diatom blooms always supported a lower zooplankton net numerical and biomass increase than simultaneous mesocosms dominated by flagellates, despite the fact that both microplankton-carbon biomass and copepod–carbon ingestion rates were similar or higher in the diatom blooms. Experimentally measured calanoid copepod egg production and hatching rates were also lower in diatom-dominated waters. In these experiments, the diet of the calanoid copepods contained diatoms when present, but the diatoms were never the preferred food.

There could also be differences in the susceptibility of different calanoid copepod species to secondary metabolites produced by diatoms. Irigoien et al. (2002) observed relatively high hatching percentages of certain calanoid species, e.g. *Calanus finmarchicus*, in the St. Lawrence estuary, even when diatoms were pres-

ent at high absolute and relative amounts (over 90 % of the microplankton). However, they also reported low values for, e.g., *Temora longicornis* and *Calanoides acutus*, at concentrations and microplankton percentages similar to those above. It is hypothesized that not all species of calanoid copepods are sensitive to toxic substances produced by diatoms. Again, the uncertainty of ingested food particles prevents a thorough analysis and discussion when evaluating papers on this subject.

The main interest in this workshop was the effect of diatoms on copepods, especially under *in situ* conditions, when different taxa of phytoplankton and protozoans (mixotrophic and heterotrophic) occur. Protozoans generally make up only a small fraction of the unicellular plankton, yet they are preferentially ingested by copepods (e.g. Kleppel et al. 1991, Fessenden & Cowles 1994) and, therefore, could directly and indirectly affect their reproduction, growth, and mortality. Also, calanoids are known to reduce their total ingestion significantly when numerous food particles are encountered, as compared to when only 1 food type is available (e.g. Paffenhöfer 1984). The reduced food intake, most likely due to food selection processes, could lessen a potential effect of diatoms, since egg viability has been shown to increase with decreasing concentrations of ingested diatom cells (Chaudron et al. 1996) and when copepods are offered mixed diets (Turner et al. 2001). Thus, one could hypothesize that many calanoid copepod species would not suffer negative diatom effects under field conditions when diatom abundances are high because of alternative choices and/or lower diatom ingestion. This could be seen as an evolutionary effect deriving from altering perceptions in response to the chemical signals provided. We are still at an early stage in determining the potential release of toxic compounds by numerous species and strains of diatoms from various parts of the oceans. Among major efforts for the near future, a priority should be the development of a catalogue or atlas of diatom species and their occurrence, so as to assess their potential toxic effect on copepods.

Nutritional value of diatoms

Most of the original studies of the nutritional quality of diatoms, as of algae in general, focused on their property to be easily ingested. In the last decade it became increasingly clear that their biochemical composition is also important for their quality as a food source for zooplankton including copepods.

Numerous studies of the fatty acid composition of diatoms have been conducted (e.g. Volkman et al. 1989, Viso & Marty 1993), while studies of other nutritional components of diatoms, such as sterols and amino acids, are relatively few (but see Volkman et al. 1998 for sterols). Most studies of the specific nutritional needs of zooplankton have concentrated on the fatty acid composition of the food. While phytoplankton can synthesize a variety of so-called essential fatty acids (ω-6 and ω-3 polyunsaturated fatty acids), amino acids and sterols, copepods cannot synthesize these compounds, and must obtain them from phytoplankton to support growth, reproduction and survival. While some calanoid species can lengthen and shorten fatty acids (Sargent & Falk-Petersen 1988), we do not know if or how it benefits survival and growth.

A recent study by Hassett (2004) showed that addition of cholesterol to a diatom diet improved both egg production and hatching viability of *Acartia hudsonica*, whereas cholesterol supplementation did not affect egg production of *Centropages hamatus*. Cholesterol supplement of different diatom diets did not always result in production improvements. Thus, there appear to be species-specific differences in diatom sterols, as well as in copepod needs for dietary sterols. It is evident that the biochemical and, thus, the nutritional composition of phytoplankton changes as the availability of dissolved nutrients changes (Morris et al. 1983, Mayzaud et al. 1989). Feeding on diatoms at different growth phases results in different copepod egg production rates and egg viability (Jónasdóttir 1994, Jónasdóttir & Kiørboe 1996). Future studies ought to simultaneously focus on more than 1 type of nutrient in order to understand the different phases of growth.

Studies of the mortality rates of developing copepod populations offered different species of phytoplankton at similar and also at different concentrations have revealed effects of food species as well as of food concentration (Paffenhöfer 1970, Carotenuto et al. 2002 and references therein). Females of certain copepod species that had been reared at high diatom concentrations were rather sluggish when escaping from hydrodynamic signals after their final molt, as compared to specimens reared on diatoms at 1 order of magnitude lower concentration (G. A. Paffenhöfer unpubl. obs.). The females receiving a higher food concentration also experienced higher and earlier mortality. No information on the effects of satiation on copepod performance appears to exist. There is also very little information on the effects of the feeding history of early juvenile stages, as well as the feeding history of parent copepods, on their present performance. By comparison, a study of several generations of humans revealed that grandparents' eating habits can affect the health of generations twice removed: 'Grandchildren of well-fed grandfathers were 4 times as likely to die from diabetes' (Kaati et al. 2002). These

effects were considered to be due to eating behavior, and not genetics.

Quantification of molecular compounds in the gut (Nejstgaard et al. 2003) may provide insight into the recent feeding history of a single copepod, especially *in situ*. The biochemical composition of the body of a copepod (Lee et al. 1971) could inform us about the feeding history on longer time scales. From those data, in relation to the food types available, we may be able to determine a copepod species' approach to a 'healthy diet'. Future nutritional studies of diatoms should include looking for biochemical differences between diatom species and changes in composition with age, to improve our insight into nutritional effects. Experimental studies should include several groups of essential nutrients. A considerable amount of information is available on the biochemical composition of phytoplankton, and numerous studies have been conducted on reproductive and behavioral responses of copepods to different types of food. This published information is seen as a foundation to be applied to future studies of the nutritional effects of diatoms and other food organisms on zooplankton.

CONCLUSIONS

The workshop was an attempt to assess the present status of research into diatom–copepod interactions, with emphasis on the effect of diatoms on copepod reproduction. We also considered the potential nutritional effects of food on growth and survival. It has to be kept in mind that the following sequence occurs in nature: a copepod encounters a wide range of potential food organisms (a spectrum), which is followed by food selection, then by the effects of the food on the copepod (whether as nauplius, copepodid stage, or adult male or female, or even through the following generations). All feeding stages are involved, and potential effects may not be highly visible or pronounced, as in some of the above-mentioned results concerning the effects of toxic diatoms on egg viability. We are at an early stage and need to chart out research plans to address the open questions and hypotheses, including those emerging as we proceed. It is clear that good communication among the research teams involved in diatom–copepod interactions will enhance progress. It is our hope that such communication will continue, reflecting the positive and collaborative attitude of the participants of our workshop.

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Determination and quantification of α,β,γ,δ-unsaturated aldehydes as pentafluorobenzyl-oxime derivates in diatom cultures and natural phytoplankton populations: application in marine field studies

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Abstract

Reactive α,β,γ,δ-unsaturated aldehydes and oxo-acids produced by marine diatoms upon cell damage interfere negatively with the reproduction success of their grazers. A simple, sensitive and specific method based on gas-chromatography coupled to mass spectrometry (EI or CI/EC) was developed for the quantification of these deleterious substances in laboratory diatom cultures and in natural phytoplankton populations. For aldehyde quantification, diatom containing samples are damaged in the presence of *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA·HCl) which leads to an in situ derivatisation without inhibition of the biosynthesis of the aldehydes. The oxime derivates of oxo-acids were in addition reacted with *N*-*tert*-butyldimethylsilyl-*N*-methyl-trifluoracetamide (MTBSTFA).
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1. Introduction

In the last decade, a chemical defence relationship mediating the interaction of diatoms and their grazers came into the focus of ecologists and chemists [1–6]. Some diatom species are able to form 2*E*, 4*E*/*Z* isomeric mixtures of α,β,γ,δ-unsaturated aldehydes like 2,4-decadienal, 2,4,7-decatrienal, 2,4-octadienal, 2,4,7-octatrienal, and 2,4-heptadienal upon cell damage [7–9]. This wound activated defensive reaction is under the control of a phospholipase A_2/lipoxygenase/hydroperoxide lyase enzyme cascade [10–12]. The fatty acid derived polyunsaturated aldehydes (PUA) were implicated in numerous deleterious effects on herbivorous crustaceans including the interference with their reproductive success by inhibiting egg hatching and the reduction of their survival [3,6]. Diatoms are dominant primary producers and key players in the marine food web [13,14]. Because of this central importance, the influence of these unicellular algae on higher trophic levels has to be investigated with emphasis on PUA-presence and function in further studies. Up to now, the metabolites in question were only determined in cultivated diatom strains [3,7–9,15], and a direct proof of the aldehyde formation in natural phytoplankton was not obtained. Nevertheless, several field studies address the relationship between diatom blooms and copepod reproduction success without giving any information about the actual occurrence of deleterious aldehydes in the plankton [3,6,16]. Phytoplankton samples contain numerous diatom species and a prediction of their PUA-production

Abbreviations: PFBHA·HCl, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride; PFBO, *O*-(2,3,4,5,6-pentafluorobenzyl) oxime; MTBSTFA, *N*-*tert*-butyldimethylsilyl-*N*-methyl-trifluoracetamide; 12-ODTE, (5*Z*,8*Z*,10*E*)-12-oxo-dodeca-5,8,10-trienoic acid; PUA, polyunsaturated aldehydes; CI/EC, chemical ionisation electron capture; TRR, *Thalassiosira rotula*; SC, *Skeletonema costatum*; PM, *Prorocentrum minimum*; AF, *Asterionella formosa*; TPA, *Thalassiosira pseudonana*

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is impossible. This is due to the fact that not all diatom species are able to produce PUA upon cell damage and that even different isolates from the same species have varying PUA-producing capability [7]. Investigation of phytoplankton field samples is mainly hampered by the fact that only few producing cells are present in a litre of seawater and that the sensitivity of the established methods for aldehyde detection is often not sufficient. Headspace extraction [15], solid phase microextraction (SPME) [10,17] and a Wittig-based-derivatisation approach of volatile aldehydes [9] were previously applied to diatom cultures for determination of these aldehydes. Given the low effective concentration of PUA causing the malfunctions in grazers [18], the Wittig-derivatisation which allows the stabilisation of reactive PUA and enables NMR analysis [9] seems to be too insensitive for the determination of PUA in phytoplankton field samples without significant concentration efforts. The more sensitive SPME approach implicates an immediate GC/MS analysis, which is often not feasible during field studies. In order to overcome the problems of low sensitivity or of low practical value during field studies, we developed an in situ derivatisation of PUA in phytoplankton matrices. The fast and sensitive method for the determination of aldehydes is based on the treatment of algal samples with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA·HCl) before wounding of the cells. In previous studies, the analysis of PFB-oxime derivatives in different biological matrices was successfully applied and provided a sensitive detection method for labile aldehydes [19–22]. The GC/MS identification of these derivates and their fragmentation by EI [23] or CI [20] is well established. We have extended the use of this derivatisation method for PUA-determination during marine field studies and for screening of diatom cultures in algal collections. The introduced protocol allows enzymatic reactions in the presence of the derivatisation reagent and thus enables an in situ trapping of aldehydes upon cell disruption of diatoms. An enzyme cascade is activated that releases PUA, which are directly converted to *O*-pentafluorobenzyl-oxime derivates (PFB-oximes). Our method combines the advantages of sensitivity of detection and stability of oxime derivates with mild derivatisation conditions that allow parallel enzymatic reactions.

2. Experimental

2.1. Reagents

2*E*,4*E*-octadienal (96%), 2*E*,4*E*-decadienal (89%), tridecanal (95%) and H_2SO_4 were purchased from Sigma (Taufkirchen, Germany). 2*E*,4*E*-Heptadienal (97%), *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA·HCl, 99%) and *N*-*tert*-butyldimethylsilyl-*N*-methyl-trifluoracetamide (MTBSTFA, 97%) were obtained from Fluka (Taufkirchen, Germany). Benzaldehyde (99%), methanol and hexane were obtained from Merck (Darmstadt, Germany).

2.2. Equipment

A Finnigan Trace GC/MS (Thermo Finnigan, CA, USA) equipped with a 15 m EC-5 capillary column (0.25 mm internal diameter, 0.25 μM film thickness, Alltech, USA) was used for EI-MS measurements. The inlet temperature was maintained at 250 °C and samples were injected in splitless mode. The column oven was held at 60 °C for 2 min, programmed from 60 to 300 °C at 8 °C/min and finally held at this temperature for 3 min. Helium was used as carrier gas at a constant flow of 1.5 ml/min and the transfer capillary was held at 270 °C. Ionisation energy was 70 eV with the ion source at 200 °C. The mass detector was operated in the TIC mode at a scan rate of 2.4 scans/s. To increase sensitivity negative ion chemical ionisation electron-capture mass spectrometry with methane as reagent gas (70 mTorr pre-vacuum) was used. This was performed on a Finnigan GC Q GC/MS (Thermo Finnigan, CA, USA) equipped with a 30 m RTX-200 column (i.d. = 0.25 mm, 0.25 μm film thickness) (Restek, USA).

2.3. Cultivation and sampling

Cultures of *Thalassiosira rotula* (TRR, CCMP 1647) and *Thalassiosira pseudonana* (TPA, CCMP 1335) were obtained from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (Maine, USA). *Skeletonema costatum* (SC, RCC 75) and *Prorocentrum minimum* (PRO, RCC 291) were obtained from the Station Biologique, Roscoff in France. The fresh water diatom *Asterionella formosa* (AF, SAG 8.95) was purchased from the Culture Collection of Algae (SAG) at the University of Göttingen (Germany).

Marine diatoms were grown in standing cultures at 16 °C in artificial medium [24] to the final concentration of 7×10^4 to 23×10^4 cells/ml (TRR). Illumination was provided on a 14:10 light:dark rhythm. *A. formosa* was cultured as described in [10]. Cells were counted with the Neubauer improved chamber (Marienfeld, Germany). Phytoplankton was sampled using a phytoplankton net (mesh size, 20 μm) in the coastal area off Roscoff (Brittany, France) during a weekly ship cruise in spring 2003 and spring 2004. Samples were gently concentrated by filtration. A filter with a mesh size of 315 μm was used to remove larger particles and zooplankton. The phytoplankton containing flow through was concentrated on an 11 μm filter. This was rinsed with 60 ml filtered seawater and residual herbivores were removed by pipetting. For cell counting, 51 ml of the initially collected seawater samples were filled into a graduated cylinder. After addition of a few drops of Lugol solution (10 g KI and 5 g I_2 in 100 ml water) the cylinder was covered and the samples were left undisturbed for 2 days before evaluation by counting sedimented cells on the grid [25].

2.4. Derivatisation and standards

Freshly sampled and prepared phytoplankton samples or cultivated diatoms were further concentrated onto a GF/C filter with a diameter of 21 or 47 mm (Whatman, GB) under reduced pressure (700 mbar). The filter was rinsed with 1 ml derivatisation reagent (25 mM PFBHA·HCl in 100 mM Tris/HCl, pH 7.0) and the solution was transferred to a 4 ml vial, which can be sealed air tight with a Teflon septum. After addition of 5 μl of internal standard (1 mM benzaldehyde in methanol), the sample was cooled to 4 °C and treated with ultra sound for 1 min (B. Braun Sonicator 1000l, Germany). The vial was sealed and incubated at room temperature for 30 min. For extraction, 0.5 ml methanol and 1 ml hexane were added [19] and the sample was vortexed for 1 min. The mixture was acidified by addition of a few drops of sulphuric acid and vortexed again. The hexane upper layer was removed by pipetting, dried over sodium sulphate and evaporated under a stream of argon. The residue was taken up in 50 μl hexane. The obtained samples can be directly used for GC/MS determination of unsaturated aldehydes with aliphatic side chains. For additional derivatisation of carboxylic groups, 10 μl MTBSTFA are added and after incubation at room temperature for 1 h, these samples can be directly analysed by GC/MS [26,27]. Derivates were identified by comparison with commercial and synthetic standards [10,28]. A one pot reductive *bis*-Wittig-olefination was applied for the synthesis of decatrienal. Therefore, propyl-1,3-*bis*-triphenylphosphonium bromide was reacted in a sequential one pot synthesis with THP-protected (*E*)-4-hydroxy-but-2-enal and propionaldehyde according to [29] the protection group of the obtained THP ether was removed using PPTS and the resulting alcohol oxidized with activated MnO_2 (Adolph, unpublished results).

2.5. Wound activation of aldehyde production and in situ derivatisation

In order to evaluate the influence of the derivatisation reagent on the enzymatic production of aldehydes and the influence of different cell damage protocols, a series of extraction protocols was tested. Three replicates of four samples of a TRR-culture (each 50 ml, 7.4×10^4 cells/ml) were filtered as described above and cell damage to activate aldehyde production [10] was performed by different approaches. Two sets of filters were rinsed either with seawater or directly with the derivatisation reagent (25 mM). After addition of the internal standard, both mixtures were treated with ultrasound. After incubation for 15 min, derivatisation reagent was added to the seawater rinsed sample (final concentration: 25 mM) and the samples were incubated at room temperature for 30 min. One further sample was rinsed with derivatisation reagent and frozen to stimulate aldehyde production [15]. After thawing, the sample was incubated at room temperature for 30 min. One filter was only rinsed with derivatisation reagent and incubated without any further treatment for 30 min.

2.6. Calibration curves and quantification

Calibration curves were determined for the major unsaturated aldehydes in the range from 0.1 up to 8.0 μM in triplicates. Therefore, different amounts of a stock solution of an aldehyde mixture (1 mM, 2E,4*E*-heptadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-decadienal, respectively, in MeOH) were added to 4 ml glass vials with 1 ml derivatisation reagent and 5 μl of internal standard (1 mM benzaldehyde in methanol). Extractions and GC EI/MS measurements were performed as described above. The calibration curves were constructed by plotting the area ratios of each analyte relative to the internal standard against the concentration of the analyte. For quantification of 2,4,7-octatrienal and 2,4,7-decatrienal, the calibration curve of the corresponding commercially available 2,4-dienals were used, which were assumed to behave similarly during derivatisation and GC/MS.

Linearity between diatom cell density and PUA-detection was verified with 50 ml of a TRR-culture, which was filtered as described above and rinsed with seawater. Samples (1 ml) of a dilution series from 10^4 to 10^6 cells/ml were directly treated with 1 ml of derivatisation reagent before sonication. The linear regression of diatom cells against aldehyde production was calculated.

2.7. Stability of the derivates

Stability of the derivates was determined by splitting a hexane extract. After immediate GC/MS analyses, one part was stored for 11 days at room temperature, the other sample was stored for the same time at −80 °C. The detected derivates in the three samples were compared.

2.8. Recovery of the derivates from phytoplankton matrices

Recovery of the derivates was determined by analysing triplicates of a phytoplankton sample spiked with the aldehyde mixture (see Section 2.6) at a final concentration of 1 μM. The measured concentrations were corrected by the natural amount of these aldehydes in the same sample. For comparison, identical amounts of aldehydes were directly derivatised.

3. Results and discussion

3.1. Derivatisation and cell damage procedures

A strain of the diatom *T. rotula* which produces 2,4-octadienal and 2,4,7-decatrienal as dominant PUA besides minor amounts of 2,4-hepatdienal, 2,4,7-octatrienal and 2,4-decadienal [7] was used for method development. From earlier work, it is known that *T. rotula* produces PUA only after cell damage [10]. In order to determine this PUA-production upon cell disruption, we have conducted a se-

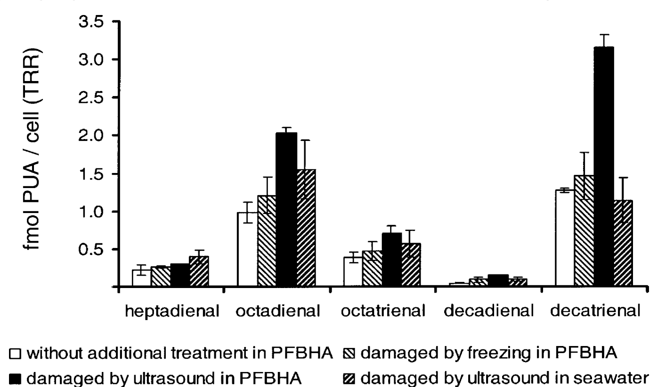

Fig. 1. Validation of different cell damage treatments. The sum of isomers are given, error bars are based on S.D. ($n = 3$). Open bars: addition of derivatisation reagent to filtered diatoms; white hatched bars: addition of derivatisation reagent to filtered diatoms with subsequent freezing and thawing; black bars: addition of derivatisation reagent to filtered diatoms with subsequent ultrasound treatment; black hatched bars: derivatisation reagent added 15 min after sonication of filtered diatom cells in seawater.

ries of different cell damage and derivatisation procedures (Fig. 1). First, we determined PUA-release by damaging the cells with ultrasound treatment in seawater before addition of the derivatisation reagent. This showed clearly that the released aldehydes can be derivatised in the seawater matrix. The yields of oxime derivates increased significantly if sonication was performed in the presence of the derivatisation reagent (Fig. 1). The increased amount of PUA-derivatives detected in these experiments demonstrates that PFBHA·HCl in Tris/HCl does not inhibit the involved enzymatic activities. Therefore, the in situ-treatment with PFBHA·HCl allows the direct trapping of the products released by a phospholipase/lipoxygenase/hydroperoxide lyase enzymatic cascade. The lower yields obtained from cells damaged before derivatisation compared to those from cells damaged in the presence of the derivatisation reagent can be attributed to the high reactivity of PUA. These aldehydes might form covalent adducts to other cell constituents like DNA or proteins [19,30] if the trapping reagent PFBHA·HCl is not present. Since even simple treatment of filtered samples with the derivatisation reagent leads to detectable amounts of PUA, it can be concluded that the osmotic stress to the cells caused by the assay reagent already initiates the formation of the aldehydes. The cell damage caused by shock freezing in liquid nitrogen and thawing of cells embedded in derivatisation reagent is convenient, but the yield of oxime derivates reaches only 55% of the total amount of PUA detected after ultrasound treatment in derivatisation reagent. To obtain best yields, we suggest to perform cell damage preferably by ultrasound directly in the derivatisation reagent under near physiological conditions at pH 7.

For development of GC/MS methods for the detection of oxo-acids, we used the 12-ODTE producing diatom *A. formosa*. Additional derivatisation of the acidic head group is required to render the analytes volatile and suitable for GC/MS analysis. For safe use in field studies, we suggest the use of the silylation reagent MTBSTFA, which is less toxic, compared to known methylation reagents. The oxo-acid containing samples obtained by the filtration/derivatisation/extraction approach as described above can be silylated and used directly for GC/MS. The silylated acids bear the additional advantage over methylated acids that the ion with a mass of M-57 ($(H_3C)_3C^{\bullet}$) can be used as analytical fragment [27].

3.2. Application in field studies and recovery of PFB-oxime derivates

During field studies the derivatisation and extraction has to be conducted under standard laboratory conditions following a simple and reproducible protocol. The applied methods should enable a safe handling without additional security precautions, which is fulfilled for the PFBO-derivatisation and the silylation. To monitor the stability of the oxime derivates we compared the detectable derivatives directly after sample preparation with those from samples that have been stored at room temperature or frozen. The derivates proved to be relatively stable (80–90% recovery after 11 days) at room temperature, which is essential for temporally not temperature-controlled storage during a field trip. Moreover, the oxime derivates did not undergo degradation when stored at −80 °C.

Due to the possible reactions of PUA with other components in the phytoplankton extract, the recovery from this matrix was determined. Therefore, defined amounts of PUA were added after filtration to phytoplankton samples. The recovery for 2,4-hepatdienal, 2,4-octadienal and 2,4-decadienal was in the range from 43 to 62%. The loss of detected PUA is probably due to binding of these reactive 2,4-dienals to nucleophiles like amino acid residues of proteins [19] or DNA [30] in the plankton sample.

3.3. GC/MS analysis of aldehyde-PFB-oxime derivatives

In order to validate the derivatisation procedure, we have chosen two important bloom forming marine diatom species *T. rotula* and *S. costatum* as well as the fresh water diatom *A. formosa*. Furthermore, we have investigated *T. pseudonana*, the first marine algae with a complete sequenced genome. *P. minimum* (PM), a dinoflagellate which does not produce any unsaturated aldehydes, served as a negative control [7]. Aldehyde-PFBO-derivates of a natural phytoplankton population, dominated by diatoms during the spring bloom in the coastal waters off Roscoff (Brittany, France) was investigated as well. In this sample, about 5500 *Thalassiosira* cells/l seawater were present and the total diatom cell count was 1.5×10^4 cells/l (Fig. 2A). The ion trace chromatograms of m/z 181 (GC EI/MS) can be used for the detection of all derivatised aldehydes in this study (Fig. 2B–E). Interestingly, the diatom TPA does not produce any of the hitherto known diatom-PUA upon cell disruption, and besides an unidentified carbonyl, GC/MS profiles were comparable to those from the negative control PM. With the cultivated diatoms TRR and SC, we could identify and quantify all PUA described in two

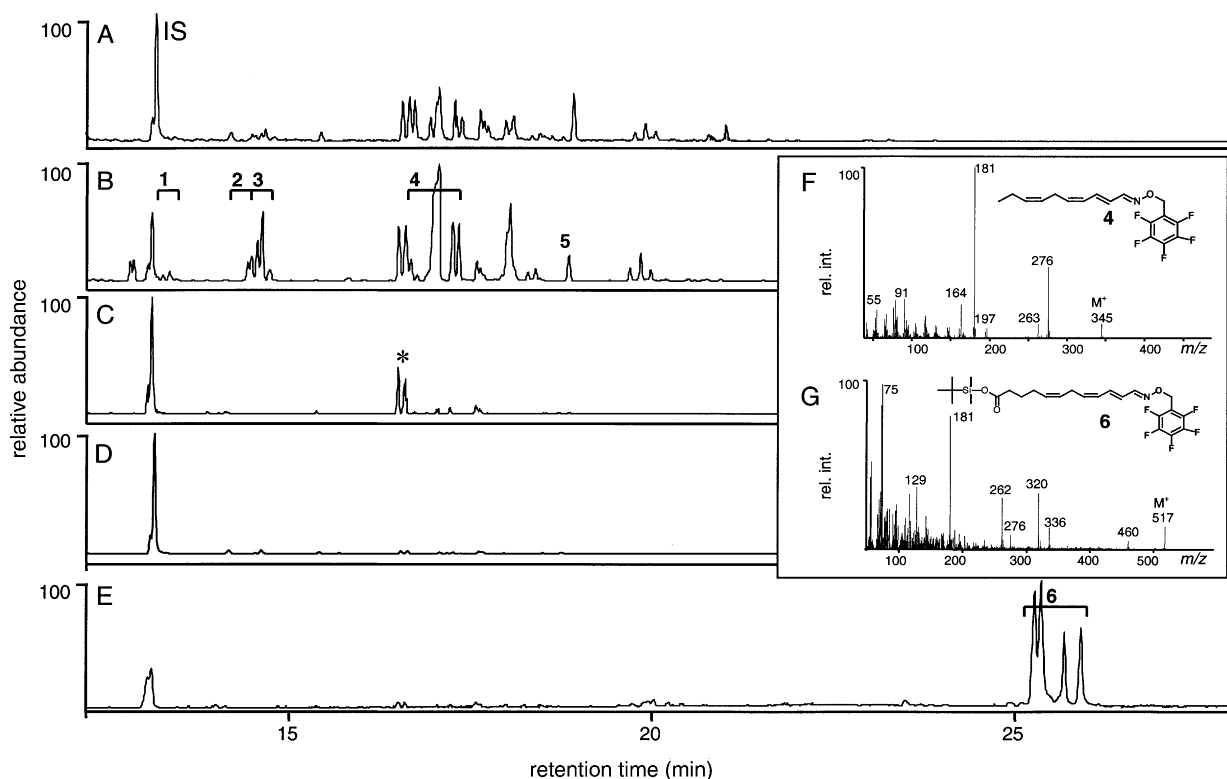

Fig. 2. Ion trace chromatograms (*m/z* 181) of phytoplankton and culture extracts: (A) phytoplankton; (B) *Thalassiosira rotula*; (C) *Thalassiosira pseudonana*; (D) *Prorocentrum minimum*; (E) *Asterionella formosa*. The numbers 1–6 correspond to the oxime derivates of the identified substances: (1) 2,4-heptadienal; (2) 2,4,7-octatrienal; (3) 2,4-octadienal; (4) 2,4,7-decatrienal and 2,4-decadienal; (5) tridecanal and (6) 12-ODTE. The insets (F and G) show mass spectra of the peak at 17.33 (4) and 25.90 (6). Analytical fragments for the PFBO-derivatives are 181 and 276 as well as the molecular ion (F and G). *tert*-Butyl-dimethylsilyl derivates prepared from acidic aldehydes show the additional characteristic fragment (M-57) due to loss of $(H_3C)_3C^{\bullet}$ (G); (*) unidentified oximes.

previous studies [7,8] as oxime derivates. We also found the derivatised saturated longer chain aldehyde tridecanal (detectable by M–OH$^{\bullet}$ and by the McLafferty fragment *m/z* 239) in phytoplankton and diatom cultures. Besides AF, neither the phytoplankton nor the other cultured species contained detectable amounts of oxo-acids.

Commercial available compounds were used to identify most of the derivates. Mass spectra of 2,4,7-decatrienal and 12-ODTE are given in Fig. 2F and G, the identity of these compounds has been proven by comparison with synthetic standards. Most of the derivates cause two or more peaks due to the syn- and anti-stereoisomers of the oximes [20]. Chromatograms are further complicated since diatoms release 2*E*, 4*E*/*Z* isomeric mixtures of α,β,γ,δ-unsaturated aldehydes [3]. Nevertheless, groups of signals, which are caused from the respective isomers of the derivatives, can be unambiguously defined by their molecular ions and fragmentation patterns. Molecular ions were also used for the quantification of the oxime derivates (Fig. 3). Standard curves were constructed for 2*E*, 4*E*-heptadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-decadienal, respectively (slopes/S.D.: 0.103 ± 0.016, $r > 0.989$; 0.120 ± 0.007, $r > 0.994$; 0.119 ± 0.011, $r > 0.991$). Linearity range of calibration curves was from 0.1 to 8.0 μM, which makes the method suitable for the quantification of PUA in phytoplankton samples as well as in few ml of laboratory cultures. Linearity was also proven between 2,4-octadienal and 2,4,7-decatrienal formation and the cell number of TRR in the range of 5×10^4 to 2.7×10^6 diatom cells (slope/S.D.: 0.117 ± 0.004, $r > 0.993$ and 0.099 ± 0.012, $r > 0.992$). The limit of quantification for the standard was 11 ng/ml medium, corresponding to less than 2000 cells of aldehyde-producing-species in 1 l seawater before filtration. For comparison, the phytoplankton blooms in French Brittany coastal waters reach 10^5 to 10^6 diatom cells per litre of seawater [31]. The detection limit of this assay can be increased by negative ion chemical ionisation electron capture mass spectrometry and detection of the $(M–HF–NO)^{\bullet -}$ ion down to 4.4 pg on column. This corresponds to less than 10 PUA-producing cells in 1 l seawater before filtration.

3.4. Quantification in cultures and phytoplankton samples

For interpretation of bioassays on the deleterious effect of diatoms on their grazers, it is essential to quantify the potential aldehyde production in the algal diets. We performed quantification in stationary phase cultures of TRR, SC and in a diatom dominated phytoplankton sample. The determined amounts of PUA from TRR fit previous reported values ob-

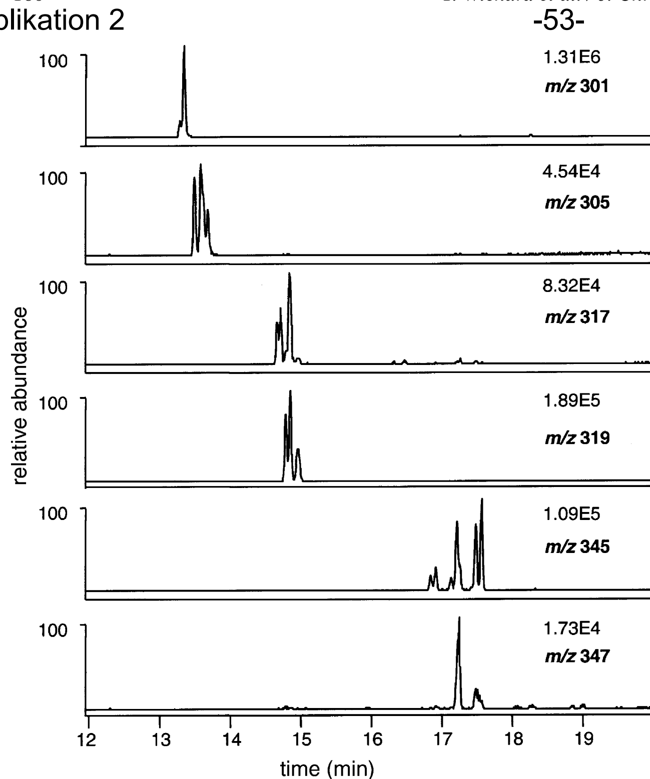

Fig. 3. Molecular ion trace chromatogram of derivatised TRR-culture extracts used for quantification. From top to bottom: the ion trace chromatograms of benzaldehyde, 2,4-heptadienal, 2,4,7-octatrienal, 2,4-octadienal, 2,4,7-decatrienal and 2,4-decadienal are shown.

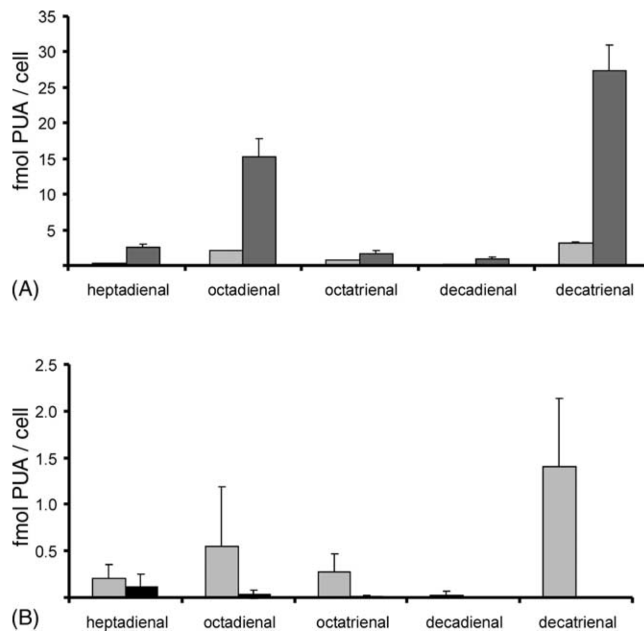

Fig. 4. Quantification of PUA in diatom cultures and phytoplankton samples. Repeated derivatisation of (A) the same TRR-culture (lighter grey, $n = 3$) and phytoplankton sample (dark grey, $n = 3$); (B) different cultures TRR (lighter grey, $n = 10$) and SC (black, $n = 4$). The error bars are based on S.D.

tained by solid phase microextraction [7]. TRR produces major amounts of 2,4-octadienal and 2,4,7-decatrienal and minor amounts of 2,4-hepatdienal, 2,4,7-octatrienal and 2,4-decadienal upon cell damage, whereas SC only forms major amounts of 2,4-hepatdienal and 2,4-octadienal [8] (Fig. 4). For estimation of the deleterious potential of a phytoplankton population, we calculate the sum of all α,β,γ,δ-unsaturated aldehydes, thus including all compounds in the sample containing the structural motive, which can be made responsible for antiproliferative effects [28]. The determination of PUA in triplicates of the same TRR-culture shows a low standard deviation and high reproducibility (Fig. 4A). The PUA-quantification from different cultures of the same strain show a higher standard deviation probably due to the varying ability of PUA-production depending on the age of the culture and to errors related to cell counts (Fig. 4B). Interestingly, the natural phytoplankton is a stronger producer than the cultivated TRR (total amount of PUA/S.D.; natural phytoplankton samples: 47.7 ± 5.9 fmol/cell, $n = 3$, compared to TRR: 6.35 ± 0.29 fmol/cell, $n = 3$). This might be due to optimum growth conditions of the field population, the bigger cell size in natural samples or increased intrinsic toxicity of field diatoms compared to the same species cultured in the laboratory. The pattern of aldehyde production formed by the diatom-dominated phytoplankton resembles that obtained from the TRR-culture. It is most likely caused by *Thalassiosira* spp. dominating the natural phytoplankton population (~35% of total diatom population) in the coastal area of Roscoff (Brittany) during the sampling period in April 2004.

4. Conclusion

We have applied a simple and sensitive approach for the quantification of deleterious aldehydes in marine phytoplankton matrices using standard GC/MS equipment. The method provides an excellent tool for field studies addressing the interaction of phytoplankton and the planktonic grazers. Since PUA are also considered as off flavours, monitoring of PUA-content in drinking water using the introduced method is a potential application as well [32]. Moreover, PFBO-based protocols can also be used for the determination of saturated aldehydes in drinking water samples [33] and thus different analytes might be surveyed using a single protocol. The method is fast and allows the screening for PUA and oxo-acids in algae collections as well as the monitoring during different growth phases of diatoms. The high sensitivity of GC EI/MS allows monitoring of the production of PUA from less than 2000 cells/l seawater following a well-established filtration procedure. The detection limit can be increased by more than two orders of magnitude if negative ion CI/EC/MS equipment is available. Derivatisation with PFBHA·HCl and MTBSTFA enables to detect both, the volatile PUA, and the acidic aldehydes harbouring a Michael-acceptor structure element. The aliphatic α,β,γ,δ-unsaturated aldehydes can be identified by three characteristic peaks in EI/MS (m/z 181, m/z 276 and the molecular ion), whereas oxo-acids are eas-

ily detectable by the fragments m/z 181 and (M-57). This approach is now routinely used for the determination of the toxic potential of diatom populations in Roscoff.

Acknowledgements

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RAPID COMMUNICATION

SURVEY OF THE CHEMICAL DEFENCE POTENTIAL OF DIATOMS: SCREENING OF FIFTY ONE SPECIES FOR $\alpha,\beta,\gamma,\delta$–UNSATURATED ALDEHYDES

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Abstract—In recent years a negative influence of diatom-derived α,β,γ,δ-unsaturated aldehydes (PUA) on the reproductive success of copepods and invertebrates has been suggested. Since adverse chemical properties of diatoms would question the traditional view of the marine food web, this defense mechanism has been investigated in detail, but the PUA-release by test organisms has only been determined in a few cases. The observed effects were nevertheless frequently discussed from a general point of view often leading to contradictory conclusions. We have examined the PUA-production of 51 diatom species (71 isolates) in order to provide a basis for the interpretation of laboratory and field results on the influence of diatom food on the reproductive success of their consumers. PUA-production is species and strain dependent. Thirty-six percent of the investigated species (38% of the cultivated isolates) release α,β,γ,δ-unsaturated aldehydes upon cell disruption in concentrations from 0.01 to 9.8 fmol per cell. *Thalassiosira rotula* and *Thalassiosira pacifica*, major spring-bloom forming diatoms

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isolated from Roscoff (Bretagne, English Channel, France) and Puget Sound (Washington, USA) were among the PUA-producing strains.

Key Words—Alga/herbivore interactions, plankton, pentafluorobenzylhydroxylamin, copepod, reproductive success

INTRODUCTION

Diatom-derived α,β,γ,δ-unsaturated aldehydes (polyunsaturated aldehydes, PUA) can interfere with the hatching success and larval development of copepods as well as with the embryonic development of invertebrates (see Ianora et al., 2003 and Pohnert, 2005 for reviews). Diatoms are unicellular algae that contribute substantially to the phytoplankton and are thus an important base of the marine food web. A generally negative impact of diatoms on herbivores would have major implications for the classical concept of plankton ecosystem functioning. Therefore, numerous studies, mainly based on laboratory experiments, have been carried out to clarify this predator-prey relationship. While some authors have proposed a deleterious effect of diatoms (Ianora et al., 2003), others have not found any adverse effects (e.g., Jonasdottir et al., 1998). Moreover, no general negative relationship between copepod egg hatching and diatom biomass was detected in the global field survey by Irigoien et al. (2002). Most of these studies correlated hatching parameters to the presence or absence of diatoms rather than monitoring PUA production of the diet, even if this is the postulated determining factor in these interactions. This is surprising, since only a few marine and fresh water diatoms have been investigated for PUA-production (Pohnert, 2005). Indeed, some of the controversially discussed results on reproductive parameters (Paffenhöfer et al., 2005) might be explained by variability of PUA production in different diatom species. To overcome this limitation and to provide a basis for further ecological and modelling studies, we conducted a survey of volatile PUA-production from representative diatoms of the major classes by using a protocol based on in situ trapping of the reactive metabolites (Wichard et al., 2005).

METHODS AND MATERIALS

Cultivation. Seventy one diatom-isolates from different sources were investigated (Table 1). All isolates were grown in stationary cultures using 250 ml jars containing 100 ml artificial medium (Table 1). Sylvania (Germany)

TABLE 1. OVERVIEW OF THE ISOLATES INVESTIGATED BY SOLID PAHSE MICROEXTRACTION AND PFBHA-DERIVATISATION

Species	SPME/ PFBHA[a]	Detected PUA	Medium[b]	Source or culture no.
Actinocyclus subtilis	•/•	–	f_2	CCAP 1000/1
Amphiphora paludosa	–/•	+[c]	f_2	SAG 15.83
Asterionella formosa	•/•	–[k]	WC	SAG 8.95
Asterionellopsis glacialis	•/•	+	f_2	PLY 607
Chaetoceros calcitrans	•/•	–	f_2	CCAP 1010/11
Chaetoceros compressus	•/•	+	f_2	PLY 550
Chaetoceros muelleri	•/•	–	f_2	CCMP 1316
Coscinodiscus granii	•/•	–	f_2	Unknown
Coscinodiscus sp.	–/•	–	f_2	RCC 773[d]
Cyclotella meneghiniana	•/•	–	WC	SAG 1020-1a
Ditylum brightwellii	•/•	–	f_2	PLY 609
Ditylum brightwellii	•/–	–	f_2	Friday Harbor[e]
Ditylum brightwellii	–/•	–	f_2	RCC 775[d]
Fragilaria capucina	•/•	–	WC	Lake Constance[f]
Fragilaria sp.	•/•	+	WC	Lake Constance[f]
Gomphonema parvulum	•/–	–[k]	WC	SAG 1032-1
Guinardia deliculata	–/•	+	K	Roscoff[d]
Guinardia striata	–/•	–	K	Roscoff[d]
Melosira nummuloides	•/•	+	f_2	CCAP 1048/6
Melosira sulcata	•/•	+	f_2	Jiaozhou Bay, China
Navicula pelliculosa	•/•	–	WC	SAG 1050-3
Navicula sallinicola	–/•	–	f_2	SAG 40.96
Navicula sp.	•/•	–	f_2	RCC 781[d]
Navicula sp.	•/–	–	K	RCC 457
Navicula transitans	•/•	–	f_2	RCC 80
Nitzschia sp.	•/•	–	f_2	RCC 782[d]
Nitzschia closteridium	–/•	–	f_2	RCC 81
Nitzschia curvilineata	–/•	–	f_2	SAG 48.91
Nitzschia frustulum	–/•	–	br	SAG 1052-2
Odontella regia	•/•	+[c]	f_2	RCC 772[d]
Odontella sinensis	•/–	–	f_2	PLY 606
Paralia sulcata	•/•	–	f_2	DML[g]
Phaeodactylum tricornutum	•/•	–	br	UTEX 646
Phaeodactylum tricornutum	•/•	–	br	SAG 1090-1a
Phaeodactylum tricornutum	–/•	–	br	SAG 1090-1b
Phaeodactylum tricornutum	•/•	–	f_2	PLY 100
Pleurosigma normanii	•/–	–	f_2	MBA[g]
Pseudonitzschia sp.	•/•	–	f_2	PLY 611
Rhizosolenia setigera	–/•	–	K	Roscoff[d]
Rhizosolenia setigera	–/•	–	f_2	CCMP 1820
Skeletonema costatum	•/•	+	f_2	RCC 75
Skeletonema costatum	–/•	+	f_2	SAG 19.99
Skeletonema costatum	•/–	+	f_2	CCMP 781
Skeletonema costatum	•/–	+	f_2	CCMP 784

TABLE 1. CONTINUED

Species	SPME/ PFBHA[a]	Detected PUA	Medium[b]	Source or culture no.
Skeletonema costatum	•/–	+	f_2	CCMP 2092
Skeletonema pseudocostatum	•/•	+	f_2	See reference[h]
Skeletonema subsalsum	–/•	+	WC	SAG 8.94
Stephanodiscus hantzschii	•/•	–	WC	Lake Constance[f]
Stephanodiscus minutulus	•/•	–	WC	SAG 49.91
Stephanophyxis turris	•/•	–[k]	f_2	DML[g]
Thalassionema nitzschioides	–/•	–	K	RCC 785[d]
Thalassiosira aestivalis	•/•	+	f_2	Dabob Bay (779)[e]
Thalassiosira anguste-lineata	•/•	+	f_2	Dabob Bay (779)[e]
Thalassiosira eccentrica	•/•	–	f_2	CCAP 1085/6
Thalassiosira minima	•/•	+	f_2	CCAP 1085/8
Thalassiosira nordenskioeldii	•/•	+	f_2	Dabob Bay (748)[e]
Thalassiosira pacifica	•/•	+	f_2	Dabob Bay (779)[e]
Thalassiosira pseudonana	•/•	–	f_2	CCAP 1085/12
Thalassiosira pseudonana	•/•	–	f_2	CCMP 1335
Thalassiosira pseudonana	–/•	–	br	SAG 1020-1b
Thalassiosira punctigera	•/–	–	f_2	Point Wells (748)[e]
Thalassiosira rotula	•/•	+	f_2	CCMP 1018
Thalassiosira rotula	•/•	+	f_2	CCMP 1647
Thalassiosira rotula	•/•	+	f_2	CCMP 1812
Thalassiosira rotula	–/•	+	f_2	RCC 776[d]
Thalassiosira rotula	–/•	+	f_2	Point Wells (805)[e]
Thalassiosira rotula	•/–	+	K	RCC 290
Thalassiosira sp.	•/–	+	K	RCC 349
Thalassiosira weissflogii	•/•	–	f_2	Unknown
Thalassiosira weissflogii	–/•	–	br	SAG 122.79
Thalassiosira weissflogii	–/•	–	f_2	RCC 76
Prorocentrum micans[i]	•/–	–	f_2	Unknown
Prorocentrum minimum[i]	•/•	–	f_2	RCC 291

"Cool white deluxe (F36W 840, 4000K)" tubes provided illumination. A light regime of 16:8 (light/dark) with 30–40 μE/m^2/sec light intensity was used. Generally, the growth temperature was 15.5°C, except for *Thalassiosira aestivalis* and *Skeletonema costatum* (CCMP 784), which were grown at 13°C and 23°C, respectively. Cultures reached the stationary phase after 2–3 wk, when 60–90 ml (10^4-10^6 cells/ml culture medium, depending on species) were harvested. The cell morphology of each culture was checked prior to harvest with light microscopy. Cells were counted with a Neubauer-improved haemocytometer in four replicates. The counting variance ranged from 10–35%.

Quantitative PUA Analysis. The cultures were harvested by filtration as described in Wichard et al. (2005). For a first rapid screening of the cultivated isolates, solid phase microextraction was performed with a polydimethylsiloxan

fiber after wounding by sonication as described in Pohnert et al. (2002). To quantify PUA release upon cell damage, a protocol based on derivatization of PUA with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamin hydrochloride (PFBHA·HCl) and subsequent GC/MS (EI) analysis was applied (Wichard et al., 2005). The limit of quantification for PUA in concentrated diatom cultures was 5 ng/ml. Each analysis was performed in triplicate.

Quantitative Chlorophyll a + c analysis. In cases where PUA were determined, the chlorophyll content of the diatom isolates was quantified as well. The extraction in 90% acetone and quantification was performed in triplicate according to the standardized method No 446.0 (U.S. Environmental protection agency: Microbiological and Chemical Exposure Research and references herein). Jeffrey and Humphrey's trichromatic equations were applied: Chlorophyll a (mg/l) = $11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630}$ and Chlorophylls $c_1 + c_2$ (mg/l) = $-1.67\ E_{664} - 7.60\ E_{647} + 24.52\ E_{630}$. Each value was corrected by the absorbance at 750 nm. The ratio of PUA to Chl a + c of identical culture batches was calculated as PUA/Chl (ppm) = PUA (μg/ml)/Chl (μg/ml) $\times$ 1,000,000.

Footnotes to Table 1

Note. A cross (+) identifies a diatom as PUA-producer. Among those, several strains were selected for quantification (see Table 2).

[a] Applied analytical method.

[b] f_2 = marine enriched medium (artificial seawater) and K = K medium (filtered seawater), see for references Pohnert et al., 2002; WC = fresh water medium; br = brackish water: f_2 medium diluted with Chu-12 medium (2 + 1), see for references Carotenuto and Lampert, 2004.

[c] Only traces of PUA were detected after PFBHA derivatisation.

[d] Strains were isolated from coastal waters off Roscoff in 2004.

[e] Strains were isolated (cruise number of research vessel "C.A. Barnes" in parenthesis) from Dabob Bay, Point Wells or Friday Harbour (Puget Sound and San Juan Island, respectively, Washington, USA) in 2001–2003.

[f] See Carotenuto and Lampert, 2004.

[g] No strain number is available.

[h] Genetically described in Pohnert et al., 2002.

[j] Dinoflagellates often used as a control diet for copepods.

[k] Acidic polyunsaturated aldehydes were detected: 12-oxo-dodeca-5,8,10-trienoic acid (*A. formosa*, *S. turris*) and 9-oxo-nona-5,7-dienoic acid (*G. parvulum*).

Abbreviations: CCAP (DML) Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory, Scotland); CCMP = Centre for Culture of Marine Phytoplankton Main, USA; PLY (MBA) = Marine Biological Association Plymouth, England; RCC = Roscoff Culture Collection, France; SAG = Culture Collection Göttingen, Germany; UTEX = The Culture Collection of Algae, University of Texas at Austin, TX, USA.

Determination of Cell Volume. Living cells were measured microscopically in planar view (minimum: 20 cells). Linear measurements were converted to cell volume using different geometric approximations: a cylinder for *Chaetoceros compressus*, *Guinardia deliculata*, *Melosira* spp., *Skeletonema subsalatum*, and *Thalassiosira* spp.; a cylinder + 2 half spheres for *Skeletonema costatum* and *Skeletonema pseudocostatum*, and a cone for *Asterionellopsis glacialis*. The carbon content was determined by the carbon to volume relationship based on the equation C (pg/cell) = 0.288 × volume$^{0.811}$ (Menden-Deuer and Lessard, 2000). The PUA to carbon ratio was calculated as PUA/C (ppm) = PUA (fg/cell)/C (fg/cell) × 1,000,000.

RESULTS AND DISCUSSION

Seventy one diatom-isolates were analyzed for PUA-formation upon cell damage by sonication. The diatoms were either obtained from algal collections or freshly isolated from coastal waters off Roscoff (48°45′ N and 3°58′ W, Bretagne, France) and during several cruises to Dabob Bay, Point Wells and Friday Harbour (47° 46.14′N and 122° 50.10′W/47° 44.63′ N and 122° 25.34′ W/48.535° N and 123.005° W, Washington, USA). A total of 50 different species was investigated, with an emphasis on the family Thalassiosiraceae and the species *Phaeodactylum tricornutum*, because these diatoms are widely used in bioassays on the reproductive success of copepods (Miralto et al., 1999; Pohnert et al., 2002; Paffenhöfer et al., 2005). Under defined culture conditions (see Method section), 27 PUA-producers were identified among the 71 isolates investigated (Table 1). Out of the PUA producers, two released the unsaturated aldehydes only in trace amounts. The PUA-production upon wounding of 20 selected isolates (18 marine and 2 freshwater) was quantified during the stationary growth phase. PUA-production ranged from 0.01 fmol PUA/cell (*Thalassiosira nordenskioeldii*) to 9.8 fmol PUA/cell (*Thalassiosira pacifica*) (Table 2). This wide range over four orders of magnitude, as well as the isolate-dependent variability of structurally different unsaturated aldehydes, reflects a high plasticity within the Bacillariophyceae.

Since the calculation of PUA per cell underestimates the aldehyde contribution of species with low cell volume but probably high cell abundance in a typical herbivore diet, the PUA to carbon (PUA/C) and the PUA to chlorophyll a + c (PUA/Chl) ratio were also calculated. With respect to the PUA/Chl and PUA/C ratios, other dominant producers, such as *Skeletonema pseudocostatum* (PUA/Chl = 40,650 ppm) or *Skeletonema costatum* (SAG 19.99, PUA/C = 488 ppm), come to the fore.

TABLE 2. QUANTIFICATION OF PUA PER CELL, PER CHLOROPHYLL A + C AND PER CARBON CONTENT (STATIONARY PHASE)

Producer of polyunsaturated aldehydes	Analysis of PUA						Analysis of Chl a + c		Analysis of carbon		
	PUA	C7:2	C8:2	C8:3	C10:2	C10:3	Chl a + c	PUA/Chl a + c	cell vol.	C/cell	PUA/C
	fmol/cell	% of total PUA					μg/10^6 cells	ppm	μm^3	pg/cell	ppm
Thalassiosira pacifica	9.81 ± 0.069	70	19	11	0	0[a]	6.58 ± 0.20	113,368	1,256	94	11,859
Melosira nummuloides	8.68 ± 0.424	7	72	0	2	19	11.7 ± 1.52	95,584	5,011	288	3,717
Thalassiosira rotula (CCMP 1647)	6.35 ± 0.289	5	32	11	2	50	3.36 ± 0.23	255,331	1,574	113	7,364
Thalassiosira rotula (origin: Roscoff)	5.69 ± 0.472	7	16	18	0	58	36.4 ± 0.16	21,573	12,972	624	1,348
Chaetoceros compressus	2.82 ± 0.635	31	12	0	8	49	3.67 ± 0.17	103,668	1,704	120	2,395
Thalassiosira aestivalis	1.54 ± 0.266	78	16	6	0	0	70.6 ± 6.96	2,462	4,630	270	525
Thalassiosira anguste-lineata	1.53 ± 0.079	69	17	14	0	0	48.5 ± 7.38	3,597	2,245	150	913
Thalassiosira rotula (origin: Point Wells)	1.27 ± 0.108	24	8	41	0	27	46.6 ± 4.50	3,459	16,406	755	212
Thalassiosira rotula (CCMP 1812)	1.04 ± 0.030	24	28	23	0	24	7.69 ± 0.55	9,672	3,497	215	588
Skeletonema pseudocostatum	0.38 ± 0.041	50	49	1	0	0	1.10 ± 0.31	40,650	186	20	2,343
Thalassiosira rotula (CCMP 1018)[c]	0.22 ± 0.048	31	45	24	0	0	8.10 ± 0.28	3,179	1,593	114	278
Guinardia deliculata	0.18 ± 0.015	100	0	0	0	0	17.4 ± 1.01	1,122	3,712	226	94
Skeletonema costatum (RCC 75)	0.13 ± 0.016	58	38	3	0	0	2.21 ± 0.11	6,623	286	28	578
Fragilaria sp.	0.10 ± 0.010	65	32	0	3	0	n.d.	n.d.	452[b]	41	252
Asterionellopsis glacialis	0.05 ± 0.005	68	28	4	0	0	6.09 ± 0.03	960	176	19	343
Thalassiosira minima	0.05 ± 0.002	23	10	0	0	61	1.16 ± 0.18	5,960	172	19	393
Skeletonema subsalsum	0.04 ± 0.014	0[a]	80	0	20	0	n.d.	n.d.	227	23	303

TABLE 2. CONTINUED

Producer of polyunsaturated aldehydes	Analysis of PUA						Analysis of Chl a + c		Analysis of carbon		
	PUA	C7:2	C8:2	C8:3	C10:2	C10:3	Chl a + c	PUA/Chl a + c	cell vol.	C/cell	PUA/C
	fmol/cell	% of total PUA					$\mu g/10^6$ cells	ppm	μm^3	pg/cell	ppm
Thalassiosira nordenskioeldii	0.01 ± 0.004	0[a]	100	0	0	0	35.2 ± 2.19	121	3,776	229	14
Melosira sulcata	0.01 ± 0.001	0	100	0	0	0	n.d.	n.d.	369	35	36
Skeletonema costatum (SAG 19.99)	0.01 ± 0.001	81	19	0	0	0	3.45 ± 0.13	260	11	2	488

Note. Species sorted by descending total amount of PUA released (fmol/cell). Valuse: mean ± SD.

[a] Traces were detected;

[b] See reference (Carotenuto and Lampert, 2004);

[c] While Pohnert et al. (2002) did not detect any PUA in this strain using solid phase microextraction, the more sensitive PFBHA-derivatisation reveals that this species has to be considered as a weak PUA-producer. Isomeric mixtures of C7:2 = 2,4-heptadienal, C8:2 = 2,4-octadienal, C8:3 = 2,4,7-octatrienal, C10:2 = 2,4-decadienal and C10:3 = 2,4,7-decatrienal were detected.

Within the Bacillariophyceae, more than half of the investigated species do not produce PUA upon wounding in the stationary growth phase. In the light of the ongoing discussion about the influence of diatoms on herbivores, this result stresses that a general PUA-mediated effect can not be assumed for any given phytoplankton bloom, but that a species and strain-specific analysis is required. Recently, the hypothesis that PUA-production could be the reason for poor copepod reproductive success during spring blooms of diatoms was proposed (Ianora et al., 2004). In this context, it is interesting to note that some of the most abundant spring-bloom forming species like *Thalassiosira* spp. (e.g. *Th. rotula* and *Th. pacifica*) release high amounts of PUA. These species were isolated from different habitats, such as the Adriatic Sea (Miralto et al., 1999), the coastal waters off Roscoff (NE Atlantic) and Dabob Bay (NE Pacific).
Because the ability to produce PUA is distributed heterogeneously in the major classes of Bacillariophyceae, one cannot predict the defensive potential of certain species. Moreover, PUA-production within different isolates of one species ranges widely, and thus case-specific chemical investigations accompanying bioassays are required. For example, the different *Thalassiosira rotula* isolates investigated release PUA in a wide range of concentrations from 0.15 to 6.34 fmol/cell. In this study, only cultures in the stationary growth phase were investigated. This culture condition was selected since it is also used in most laboratory investigations. Additional variation of PUA-production during different phases of diatom blooms or growth phases of cultures might have to be taken into account as well.

Based on this survey, we not only recommend performing future bioassays along with chemical analyses, but also urge for a reconsideration of the general conclusions drawn in the past. It is likely, that the observed reduction of hatching success in several studies/regions may not be due to the formation of deleterious PUA, but may have other causes. On the other hand, in regions where major PUA producers are the main constituents of blooms, there might be effects on the reproduction of herbivorous grazers and their population dynamics (Ianora et al. 2004; Halsband-Lenk et al. unpublished). Whether secondary production can be significantly affected by this chemically mediated interaction in such ecosystems requires further investigation.

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Short synthesis of labeled and unlabeled 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid as metabolic probes for biosynthetic studies on diatoms

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Abstract

We describe a short synthesis of the unusual polyunsaturated 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid found in marine and fresh water diatoms. Using a one pot reductive bis-Wittig-olefination, the homoconjugated tetraene backbone of the fatty acid can be generated from easy available precursors. Reductive olefination allows the non-statistical dissymmetrisation of a symmetrical bis-Wittig salt as key synthon. This short sequence was also applied to the generation of the corresponding 9,10-[$^2H_2$] labeled fatty acid. If administered to cell fragments of *Thalassiosira rotula* 9,10-[$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid is transformed oxidatively to the aldehyde 1,2-[$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal which is involved in the chemical defense of this alga. Using the synthetic standard it could be shown that the C16:4 ω1 fatty acid is released upon wounding of *T. rotula* cells. The synthesis with the labeled bis-Wittig salt is of general use and can also be applied for the fast generation of other internally labeled functionalized and non-functionalized polyunsaturated fatty acids. To our knowledge this represents the first synthesis of 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid.
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Keywords: One-pot synthesis; bis-Wittig reaction; Homoconjugated tetraenes; Deuterium labeled; Unsaturated fatty acids; Reductive olefination

1. Introduction

6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (C16:4 ω1) has initially been detected in fish oil (Silk and Hahn, 1954) and was later found as an important constituent in phytoplankton samples (Klenk and Eberhagen, 1962). It is a common fatty acid in diatoms and, can therefore, be used as a trophic marker in the marine food chain (Ackman and Tocher, 1968; Dunstan et al., 1994). Recently, this fatty acid was also identified from fresh water diatom biofilms as toxic principle against the anostracan grazer *Thammocephalus platyurus* (Jüttner, 2001). Despite this importance as yet no synthesis of this unusual fatty acid was reported. This is at least partially due to the fact that the synthesis of homoconjugated tetraene systems, like it is found in C16:4 ω1 requires long sequences involving numerous coupling and protection steps (see

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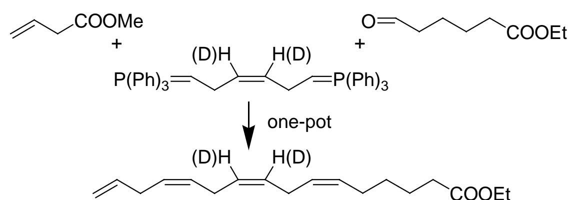
Durand et al., 2000 for a review). Here we show that a one-pot bis-Wittig sequence, recently established in our lab for the production of homoconjugated dienes and trienes (Pohnert and Boland, 2000; Pohnert et al., 1999) can be extended for the generation of the homoconjugated tetraenoic acid C16:4 ω1 (Scheme 1).

Besides the direct toxicity of free polyunsaturated fatty acids, also unsaturated aldehydes derived from fatty acids are discussed to play a key role in diatom defense (Ianora et al., 2003; Miralto et al., 1999). After cell disruption these unicellular algae release a complex bouquet of saturated and unsaturated aldehydes (Pohnert, 2000). Among these, the α,β,γ,δ-unsaturated aldehydes act antiproliferatively against the eggs of herbivorous copepods and sea urchins (Adolph et al., 2003; d'Ippolito et al., 2002b; Miralto et al., 1999). Most of the identified unsaturated aldehydes are derived from the lipoxygenase-mediated transformation of eicosanoic fatty acids (Pohnert and Boland, 2002), but recently, 2*E*,4*E*/*Z*-octadienal was reported as an exception to be derived of ω4-hexadecatrienoic acid (d'Ippolito et al., 2003). d'Ippolito et al. suggested C16:4 ω1 to be a possible precursor of the unusual metabolite 2*E*,4*E*/*Z*,7-octatrienal but experimental evidence is still lacking. Our synthetic concept of bis-Wittig olefination can be exploited for the generation of position specific labeled metabolic probes that allowed to show that 9,10-[$^2H_2$]-C16:4 ω1 is the precursor of the unusual metabolite 1,2-[$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal.

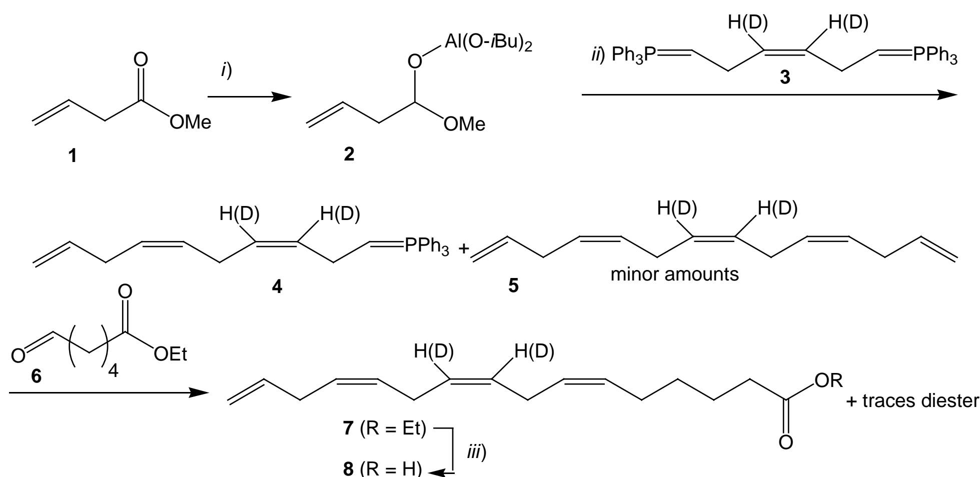
2. Results and discussion

Based on a previously introduced synthesis of homoconjugated dienes and trienes we developed a short synthesis of C16:4 ω1. Using the symmetrical bis-Wittig salt **11** (Pohnert and Boland, 2000), which was prepared from the mono THP-protected (*Z*)-hex-3-ene-1,6-diol (**9**) the homoconjugated tetraene was generated in a sequential one pot procedure. To avoid the statistical distribution of wanted unsymmetrical and unwanted symmetrical side-products arising out of the reaction of the symmetrical bis-ylide **3**, dissymmetrisation was required. This can be achieved by generating the first aldehyde equivalent in situ through diisobutyl aluminium hydride (DIBALH) reduction of the corresponding ester.



Scheme 1. Schematic representation of the formation of homoconjugated tetraenes using the bis-Wittig reaction.

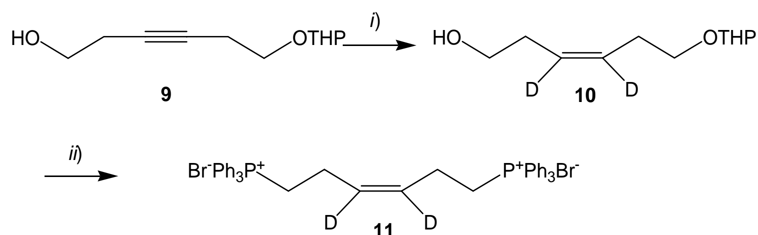
We selected the commercial available methyl 3-butenoate (**1**) as first aldehyde equivalent which also delivers one homoconjugated double bond. This was reduced by slowly adding pre-cooled DIBALH at −78 °C (Scheme 2). Meanwhile the bis-Wittig salt **11** was deprotonated to the bis-ylide **3** with 2.2 equivalents of $KN(SiMe_3)_2$. The aluminate reaction mixture was then transferred directly via a pre-cooled cannula to the bis-ylide and the mixture was allowed to warm slowly to 0 °C. During this procedure the aluminum-complex **2** decomposes and releases slowly but-3-enal that reacts preferentially with the more reactive bis-ylide **3** compared to the mono-ylide **4**. The reaction mixture was re-cooled to −30 °C and 1.2 equivalents of the second aldehyde equivalent, ethyl 6-oxo-hexanoate **6** were added. After warming to room temperature the reaction mixture was worked up as described in the material and methods section to give ethyl 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate (**7**) in 24% yield. The symmetrical by-products were detected in minor amounts of 1:0.35:0.05 (**7**:(4*Z*,7*Z*,10*Z*)-tetradeca-1,4,7,10,13-pentaene (**5**):(6*Z*,9*Z*,12*Z*)-diethyl octadeca-6,9,12-triendioate (GC-MS)). ^{13}C NMR analysis showed that the newly generated double bonds of **7** were of >95% *Z*-geometry, and the double bond geometry of the bis-Wittig-salt **11** (>98% *Z*) was retained during the reaction. This method for the generation of homoconjugated tetraenes allows thus the fast generation of highly unsaturated products from simple starting materials in a one-pot reaction. Compared to other known routes to related highly unsaturated products which are often based on sequential alkyne coupling reactions followed by a final reduction step of the generated polyyne the present approach reduces the amount of reaction steps and use of protecting groups to a minimum.



Scheme 2. Preparation of 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**). (i) DIBAL-H, (ii) from **11** by deprotonation with 2.2 equivalents of $KN(SiMe_3)_2$, (iii) LiOH/THF/H_2O.

Another advantage of this short sequence is that isotope labels can be flexibly introduced into specific parts of the target molecule. We reasoned that the deuterated bis-Wittig salt would be useful for the introduction of internal labels in the homoconjugated polyene segment. The labeled bis-Wittig salt was generated after a modified procedure reported for the generation of the unlabeled analogue (Pohnert and Boland, 2000). Mono THP-protected hex-3-yne-1,6 diol (**9**) (Eya et al., 1990) was reduced with D_2 using in situ generated P-2-nickel as catalyst (Brown and Ahuja, 1973). The resulting mono THP-protected [3,4-$^2H_2$]-(3*Z*)-hex-3-ene-1,6-diol (**10**) was transformed directly without deprotection into the labeled bis-Wittig **11** salt using Br_2/PPh_3 (Scheme 3). Compared to the published procedure (Pohnert and Boland, 2000) this direct transformation bears the advantage that the work-up of the polar intermediate hex-3-ene-1,6-diol can be avoided, but lower yields using the mono-protected diol have to be considered. The labeled bis-Wittig salt **11** was transformed as described above to ethyl [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate in 27% yield.

The labeled bis-Wittig salt **11** can be obtained in large batches and stored for a prolonged time. Since it can be also employed to flexibly generate other metabolic probes where internal label of (functionalized) unsaturated fatty acids is required, it provides a useful synthetic tool. The tolerance of aldehydes of varying chain lengths and degree of unsaturation and of functional groups now extends the synthetic approach beyond the generation of homoconjugated trienes. Established syntheses of labeled homoconjugated polyenes involve often the deuteration of corresponding polyynes (see, e.g. d'Ippolito et al., 2003).



Scheme 3. Preparation of [3,4-$^2H_2$]-(*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**). (i) P2-Ni/D_2, (ii) 1: Br_2, PPh_3; 2: PPh_3 reflux.

This results in uniformly labeled double bonds, which often make mass spectrometric investigations difficult. In contrast, our synthesis provides the advantage, that labels can be introduced in selected internal positions of the homoconjugated polyene.

The availability of [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid (**8**) allowed to investigate whether this fatty acid is a precursor for defensive metabolites in the diatom *Thalassiosira rotula*. This diatom is known to produce a variety of α,β,γ,δ-unsaturated aldehydes upon cell disruption, that contribute to its chemical defense (Adolph et al., 2003; d'Ippolito et al., 2002a; Miralto et al., 1999; Pohnert, 2000; Pohnert et al., 2002). In this alga 2*E*,4*Z*-decadienal and 2*E*,4*Z*,7*Z*-decatrienal are derived from the lipoxygenase-mediated transformation of arachidonic and eicosapentaenoic acid, respectively (Pohnert, 2002). To verify if shorter chain length fatty acids are transformed to α,β,γ,δ-unsaturated aldehydes as well, we applied [9,10-$^2H_2$]-6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid **8** to mechanically wounded *T. rotula*. Transformation products were enriched during the first 10 min after wounding using solid phase microextraction (SPME) and products were monitored with GC-MS (Fig. 1).

The labeled fatty acid was transformed with high efficiency, resulting in strongly increased levels of [1,2-$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal (**12**). The specific labeling of the fatty acid combined with the analysis of the fragmentation pattern of the mass spectrum allowed to show, that the intact C9–C16-terminus of the fatty acid is incorporated into [1,2-$^2H_2$]-2*E*,4*E*/*Z*,7-octatrienal without loss of the label at C9. This product formation is in accordance with a lipoxygenase/lyase mechanism in which molecular oxygen is introduced in 9-position of the fatty acid, followed by the action of a lyase that releases 2*E*,4*E*/*Z*,7-octatrienal (**12**) and a second yet unidentified C8-fragment (Fig. 1). The diatom *T. rotula* has thus the ability to transform both, polyunsaturated C20 and C16 fatty acids. In contrast, *Skeletonema costatum* which was shown to produce octadienal from 6*Z*,9*Z*,12*Z*-hecodecatrienoic acid lacks the activity for the formation of C10 aldehydes (d'Ippolito et al., 2002a; d'Ippolito et al., 2003). Since fatty acid addition results in a pronounced increase of labeled metabolites, the substrate availability seems to determine if C10- or C8-unsaturated aldehydes are preferentially generated as wound activated defense by *T. rotula*. This is also reflected by the fact that intact *T. rotula*, which does not contain any 2*E*,4*E*/*Z*,7-octatrienal (**12**) lacks also free C16:4 ω1 (Fig. 1C). Only upon wounding of the alga C16:4 ω1 is released from lipids and is available as precursor for the unsaturated aldehyde (Fig. 1D). The wound activated production of 2*E*,4*E*/*Z*,7-octatrienal (**12**) as well as of the unsaturated C10 aldehydes (Pohnert, 2002) is thus controlled through the release of free fatty acids upon cell disruption.

3. Materials and methods

3.1. General remarks

Reactions were performed under Ar. Solvents were dried according to standard methods. ^1H and ^{13}C NMR: Bruker Avance DRX 500 or AV 400 spectrometer. Chemical shifts of ^1H and ^{13}C NMR are given in ppm (δ) downfield relative to TMS. GC-MS: Finnigan Trace MS equipped with an Alltech EC5 column (i.d.: 0.25 mm, 0.25 μm film thickness, 15 m), Helium as carrier. HR-MS: Micromass MasSpec (Micromass, Manchester, UK). Preparative column chromatography was performed on Florisil (Sigma), or SiO_2 (ICN) 32–63, 60 Å. Reagents and solvents were purchased from Aldrich, Merck and Fluka.

3.1.1. Ethyl 6Z,9Z,12Z,15-Hexadecatetraenoate (7)

3.1.1.1. Preparation of the bis-ylide. 0.5 g (0.65 mmol) (*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**) was suspended in 25 ml THF and cooled to −78 °C before addition of 1.4 ml of 28 ml a 0.5 M solution $KN(SiMe_3)_2$ (1.4 mmol) in hexane. The suspension was warmed to −20 °C until no further deepening of the dark red color could be observed (20 min). The solution was re-cooled to −78 °C.

3.1.1.2. Preparation of the aluminate. To a cold (−78 °C) solution of methyl but-2-enoate (**1**) (65 mg, 0.65 mmol) in ether (3 ml), pre-cooled (−78 °C) DIBALH (0.65 ml of a 1 M solution in hexanes, 0.65 mmol) was added drop wise.

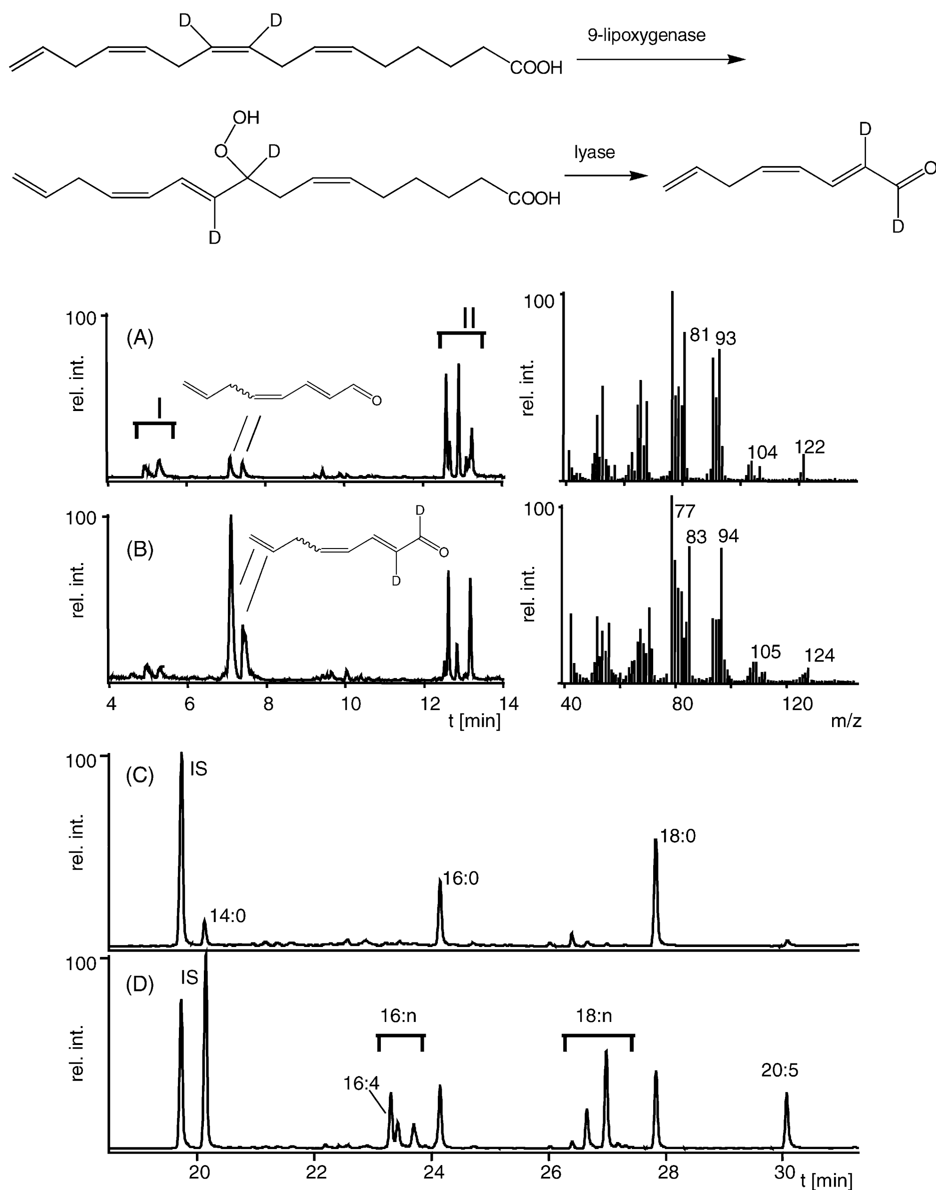


Fig. 1. Transformation of [9,10-$^2H_2$]-6Z,9Z,12Z,15-hexadecatetraenoic acid (**8**) by suspensions of damaged *T. rotula* in seawater. Above: Mechanistic suggestion for the lipoxygenase-mediated transformation of [9,10-$^2H_2$]-6Z,9Z,12Z,15-hexadecatetraenoic acid (**8**). (A) Gas chromatogram of SPME extracted volatiles from damaged *T. rotula* (I: 2*E*,4*E*/*Z*-heptadienal; II: 2*E*,4*E*/*Z*-decadienal and 2*E*,4*E*/*Z*,7*Z*-decatrienal, 2*E*,4*E*/*Z*-octadienal at RT, 7.4 min) and EI/MS of 2*E*,4*E*/*Z*,7-octatrienal (**12**). (B) as (A) after addition of 100 μg [9,10-$^2H_2$]-6Z,9Z,12Z,15-hexadecatetraenoic acid (**8**). (C) Free fatty acids in intact *T. rotula* IS: [$^2H_{27}$]-myristic acid. (D) Free fatty acids in wounded *T. rotula*.

After being stirred for 10 min (GC control) the cold (−78 °C) aluminate **2** was transferred quickly via a pre-cooled cannula to the bis-ylide reaction mixture. The mixture was allowed to warm to RT over a period of 60 min, and stirring was continued for 30 min before re-cooling to –78 °C. Then, a solution of ethyl 6-oxo-hexanoate (**6**) (110 mg, 0.7 mmol) in 5 ml THF was added. The mixture was allowed to

reach room temperature and stirred for 30 min. Hydrolysis with HCl (2 N), extraction with ether, drying over Na_2SO_4, and flash chromatography on silica gel gave ethyl 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate (**7**) in 24% yield. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.25$ (t, $J = 7.05$ Hz, 3H, O–CH_2CH_3); 1.38 (quint., $J = 7.7$ Hz, 2H, H–C3); 1.6 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.1 (dt., $J = 7.4$, 7.2 Hz, 2H, H–C5); 2.3 (t, $J = 7.5$ Hz, 2H, H–C2); 2.8 (m, 6H, C8, C11, C14); 4.1 (quart., $J = 7.1$ Hz, 2H, O–CH_2CH_3); 5.0 (dquart., $J = 10.0$ Hz, 1.6, 1H, C16); 5.05 (dquart., $J = 17.2$, 1.7 Hz, 1H, C16); 5.3–5.4 (m, 6H, C6, C7, C9, C10, C12, C13), 5.8 (m, 1H, C15). ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 14.66$, 25.02, 25.97, 26.03, 27.28, 29.49, 31.92, 34.66, 60.6, 115.15, 127.46, 128.39, 128.51, 128.76, 129.37, 130.1, 137.14, 174.11. The signals at 131.06 and 129.1 were used to determine the configurational purity of the product. EI-MS (70 eV): 276 (0.7), 247 (1), 235 (3), 208 (5), 189 (7), 147 (20), 133 (31), 119 (27), 105 (45), 91 (60), 79 (100), 67 (40).

3.1.2. [3,4-$^2H_2$]-(*E*)-1-(Tetrahydro-2H-pyran-2-yloxy)hex-3-en-6-ol (**10**)

2.5 g (10.1 mmol) $Ni(Ac)_4 \cdot (H_2O)_4$ are suspended in 81 ml ethanol (95%) and the reaction vessel was filled with deuterium. The suspension was treated with 382 mg (10.1 mmol) $NaBH_4$ in 10 ml ethanol. After 1 min at RT 2 ml ethylenediamine (30.3 mmol) are added. To the catalyst mixture 16 g (80.7 mmol) 1-(tetrahydro-2H-pyran-2-yloxy)hex-3-yn-6-ol (**9**) was added and stirred over night under D_2 atmosphere. The reaction mixture was filtered over celite and the filtrate was diluted with water. After extraction with Et_2O, drying over Na_2SO_4, removal of the solvent and column chromatography on silica gel [3,4-$^2H_2$]-(*Z*)-1-(tetrahydro-2H-pyran-2-yloxy)hex-3-en-6-ol (**10**) was obtained in 82.1% yield. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.4$–1.65 (m, 5H, H–C2′, 2H–C3′, 2H–C4′); 1.75–1.85 (m, 1H, H–C2′); 2.24 (t, $J = 6.42$ Hz, 2H, H–C5); 2.32 (t, $J = 6.79$ Hz, 2H, H–C2); 3.63–3.82 (m, 2H, H–C6); 3.28–3.45 (m, 2H, C1); 3.45–3.52 (m, 2H, H–C5′); 3.38 (s, 1H, –OH) 4.55 (t, $J = 3.21$ Hz, 1H, C1′). ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 20.3$, 26.7, 29.0, 31.7, 32.3, 62.1, 62.6, 67.6, 99.2, (127.9, 128.2, 128.3, 128.4, 128.5, 128.7, 128.8, C3, C4). EI-MS (70 eV): 202 (0.1), 172 (1), 101 (8), 85 (100), 82 (10), 67 (7), 57 (11).

3.1.3. [3,4-$^2H_2$]-(*Z*)-Hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**)

A suspension of triphenylphosphine (27.3 g, 104 mmol) in benzene (300 ml) was treated at 0 °C with bromine (16.6 g, 104 mmol). After being stirred for 10 min at room temperature, the suspension is recooled to 0 °C and [3,4-$^2H_2$]-(*Z*)-1-(tetrahydro-2H-pyran-2-yloxy)hex-3-en-6-ol (**10**) (10 g, 50 mmol) are added. After additional stirring for 2 h at room temperature, the reaction mixture was poured into petrol ether and triphenylphosphine oxide was filtered off. Crude [3,4-$^2H_2$]-(*Z*)-1,6-dibromo-hex-3-ene that was transferred without further purification into a solution of triphenylphosphine (29 g, 110 mmol) in acetonitrile (200 ml). After reflux for 5 days the mixture was poured in toluene (500 ml) and the resulting bis-Wittig-salt was filtered off. Recrystallization from methanol–ether afforded pure crystals of [3,4-$^2H_2$]-(*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**) in 39% yield. ^{1}H NMR ([D_4] MeOH, 400 MHz): $\delta = 2.2$ (m, 4H), 3.41 (m, 4H), 7.98–7.13 (m, 2H), 7.6–7.83 (m, 28H). ^{13}C NMR ([D_4] MeOH, 100 MHz): $\delta = 21.8$ (d, $J = 15.4$ Hz), 22.8 (d, $J = 49.9$ Hz), 119.9 (d, $J = 86.6$ Hz), 139.9 (d, $J = 71.9$ Hz), 131.9 (d, $J = 13.2$ Hz), 135.2 (d, $J = 10.2$ Hz), 136.9 (d, $J = 2.9$ Hz). IR (KBr) ν: 2897, 2857, 2751, 1584, 1482, 1434, 1319, 1111, 992 cm^{-1}.

3.1.4. [9,10-$^2H_2$]-Ethyl 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoate (**7**)

Prepared as described for the unlabeled **7** from [3,4-$^2H_2$]-(*Z*)-hex-3-enyl-1,6-bis-[triphenylphosphonium bromide] (**11**) 1 g (1.3 mmol). Yield: 27%. ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 1.24$ (t, $J = 7.05$ Hz, 3H, O–CH_2CH_3); 1.38 (quint., $J = 7.7$ Hz, 2H, H–C3); 1.6 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.1 (dt., $J = 7.4$, 7.2 Hz, 2H, H–C5); 2.3 (t, $J = 7.5$ Hz, 2H, H–C2); 2.8 (m, 6H, C8, C11, C14); 4.1 (quart., $J = 7.1$ Hz, 2H, O–CH_2CH_3); 5.0 (dd, $J = 10.0$ Hz, 1.6, 1H, C16); 5.05 (dquart., $J = 17.2$ Hz, 1.7, 1H, C16); 5.3–5.4 (m, 4H, C6, C7, C12, C13), 5.7 (m, 1H, C15). ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 14.66$, 25.02, 25.97, 26.03, 27.28, 29.49, 31.92, 34.66, 60.6, 115.15, 127.46, 128.51, 129.37, 130.1, 137.14, 174.11. EI-MS (70 eV): 278 (0.8), 249 (1), 237 (3), 210 (7), 191 (9), 149 (40), 135 (49), 121 (56), 107 (71), 92 (90), 80 (100), 67 (87).

*3.1.5. 6Z,9Z,12Z,15-Hexadecatetraenoic acid (**8**)*

The free acid was prepared from the ethyl ester **7** (10 mg, 0.036 mmol) by stirring in 4 ml of a 3:1 mixture THF:H_2O in the presence of LiOH (2.4 mg, 0.1 mmol) over night at room temperature. THF was removed under reduced pressure and pH was adjusted to 1 with dil. HCl. Extraction with Et_2O (3×) and column chromatography on SiO_2 gave **8** in 84% yield. ^{1}H NMR (400 MHz, [D$_4$] MeOH) $\delta = 1.32$ (quint., $J = 7.4$ Hz, 2H, H–C3); 1.51 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.1 (dt, $J = 6.6, 7.5$ Hz, 2H, H–C5); 2.2 (t, $J = 7.3$ Hz, 2H, H–C2); 2.72 (m, 4H, C8, C11); 2.83 (m, 2H, C14), 5.0 (dd, $J = 10.2, 2.0$ Hz, 1H, C16); 5.1 (dd, $J = 16.7, 2.2$ Hz, 1H, C16); 5.2–5.4 (m, 4H, C6, C7, C12, C13), 5.7 (m, 1H, C15). ^{13}C NMR (100 MHz, [D$_4$] MeOH) $\delta = 26.11$, 28.27, 30.26, 30.55, 31.3, 32.88, 35.21, 115.47, 128.42, 129.09, 129.6, 129.75, 130.42, 131.46, 138.31, 177.92.

*3.1.6. [9,10-$^2H_2$]-6Z,9Z,12Z,15-Hexadecatetraenoic acid (**8**)*

[9,10-$^2H_2$]-**8** was prepared as described for **8** from [9,10-$^2H_2$]-ethyl 6Z,9Z,12Z,15-hexadecatetraenoate (**7**) (10 mg, 0.036 mmol) in 82% yield. ^{1}H NMR (400 MHz, [D$_4$] MeOH) $\delta = 1.31$ (quint., $J = 7.6$ Hz, 2H, H–C3); 1.52 (quint., $J = 7.7$ Hz, 2H, H–C4); 2.0 (dt, $J = 6.5, 7.4$ Hz, 2H, H–C5); 2.2 (t, $J = 7.3$ Hz, 2H, H–C2); 2.7 (m, 6H, C8, C11, C14); 4.9 (d, $J = 10.2$ Hz, 1H, C16); 4.95 (d, $J = 17.0$ Hz, 1H, C16); 5.2–5.4 (m, 6H, C6, C7, C12, C13), 5.8 (m, 1H, C15). ^{13}C NMR (100 MHz, [D$_4$] MeOH) $\delta = 26.09$, 28.22, 30.26, 30.54, 31.3, 33.87, 35.21, 117.91, 128.41, 129.09, 129.6, 131.46, 138.34, 177.96. EI-MS (70 eV): 250 (0.3), 209 (5), 182 (7), 149 (10), 135 (14), 121 (21), 107 (35), 92 (74), 80 (100), 67 (52).

*3.1.7. Transformation of [9,10-$^2H_2$]-6Z,9Z,12Z,15-hexadecatetraenoic acid (**8**) by T. rotula*

Twenty microliters of a cultured *T. rotula* in the late logarithmic growth phase (for details see Pohnert, 2002) were concentrated by centrifugation to give 2 ml of a dense cell suspension. The cells were transferred to 5 ml glass vials that can be sealed air tight with a Teflon cap. Ten microliters of a 10 mg ml^{-1} solution of [9,10-$^2H_2$]-6Z,9Z,12Z,15-hexadecatetraenoic acid (**8**) in MeOH were added and the set up was sonicated in an ice bath with four 80 W, 5 s pulses of a 1000L Sonicator (B. Braun Biotech, Melsungen, Germany). The samples were directly sealed after sonication and a polydimethylsiloxane-coated (100 μm) SPME fiber (Supelco, Bellefonte, PA) was introduced in the headspace over the medium. Extraction was performed for 10 min at room temperature. Evaporation of the volatiles from the fiber was directly performed within the injection port (250 °C) of the GC-MS (*T* program: 50 °C [2 min, splitless] ramped with 10 °C min^{-1} to 200 °C and then with 30 °C min^{-1} to 280 °C). Unsaturated aldehydes were identified as described (Adolph et al., 2003). Free fatty acids in intact and wounded *T. rotula* were determined as described elsewhere (Pohnert, 2002), 6Z,9Z,12Z,15-hexadecatetraenoic acid was identified by co-injection with the synthetic standard; GC-MS (*T* program: 60 °C [2 min, split 1:20] ramped with 5 °C min^{-1} to 300 °C (2 min)).

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Aldehyde suppression of copepod recruitment in blooms of a ubiquitous planktonic diatom

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The growth cycle in nutrient-rich, aquatic environments starts with a diatom bloom that ends in mass sinking of ungrazed cells and phytodetritus[1]. The low grazing pressure on these blooms has been attributed to the inability of overwintering copepod populations to track them temporally[2]. We tested an alternative explanation: that dominant diatom species impair the reproductive success of their grazers. We compared larval development of a common overwintering copepod fed on a ubiquitous, early-blooming diatom species with its development when fed on a

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typical post-bloom dinoflagellate. Development was arrested in all larvae in which both mothers and their larvae were fed the diatom diet. Mortality remained high even if larvae were switched to the dinoflagellate diet. Aldehydes, cleaved from a fatty acid precursor by enzymes activated within seconds after crushing of the cell[3], elicit the teratogenic effect[4]. This insidious mechanism, which does not deter the herbivore from feeding but impairs its recruitment, will restrain the cohort size of the next generation of early-rising overwinterers. Such a transgenerational plant–herbivore interaction could explain the recurringly inefficient use of a predictable, potentially valuable food resource—the spring diatom bloom—by marine zooplankton.

The dense phytoplankton blooms that characterize productive regions and seasons in the sea are dominated, from high to low latitudes and from coast line to open ocean, by comparatively few, often cosmopolitan species of diatom[5]. A significant fraction of their biomass sinks out ungrazed, because the populations of their main grazers—calanoid copepods—generally peak well after the bloom, under much lower food concentrations than during the bloom[5,6,7]. This paradox has been attributed to the slow response times of copepod life cycles, which involve 11 larval stages and take weeks to months (depending on temperature) to complete[8]. However, the faster-growing protistan grazers of diatoms are also unable to keep track of the bloom. This contrasts strikingly with the ubiquitous microbial food-webs, in which heavy grazing pressure restricts population build-up of all components[9]. Indeed, the bloom-forming diatoms differ from other unicellular plankton in their ability to dominate total pelagic biomass. Clearly, they are less grazed than other species.

Copepod life-cycle strategies differ widely, from year-round reproductive activity to strong seasonality interspersed with dormant stages of varying length[8]. Yet none of the species reaches its annual peak abundance during diatom blooms, suggesting that diatoms are not an ideal food source. A negative effect of diets of various diatom species on copepod egg hatching success (up to 100%) has been demonstrated experimentally[10,11], although its ecological implications remain controversial[12]. To test whether maternal and post-embryonic diatom diets also affected larval development and survival, we carried out experiments in which pure cultures of the cosmopolitan, coastal and oceanic bloom-dominating diatom *Skeletonema costatum* (SKE) were fed to *Calanus helgolandicus*, a dominant copepod in the temperate Atlantic. Controls were run with a small dinoflagellate, *Prorocentrum minimum* (PRO), typical of the bloom aftermath stage. We also followed the development of nauplii spawned from wild females collected during the recurrent winter/spring diatom bloom in the North Adriatic Sea[11], where *C. helgolandicus* individuals, overwintering at depth, rise to the surface and start first-feeding from late winter to early summer[13].

C. helgolandicus females were conditioned with PRO for 24 h and then were either kept on this diet (control) or switched to a diet of SKE. Eggs were collected after three, five and seven days of feeding and allowed to hatch. Offspring were then raised on either PRO or SKE so that combinations of diets were tested: those in which both mothers and progeny always fed on either SKE or PRO, and those in which mothers received either PRO or SKE, and neonates were switched to the other diet.

Maternal and neonatal diet of SKE (SKE/SKE) resulted in 100% larval mortality, but the larval stage reached by the offspring

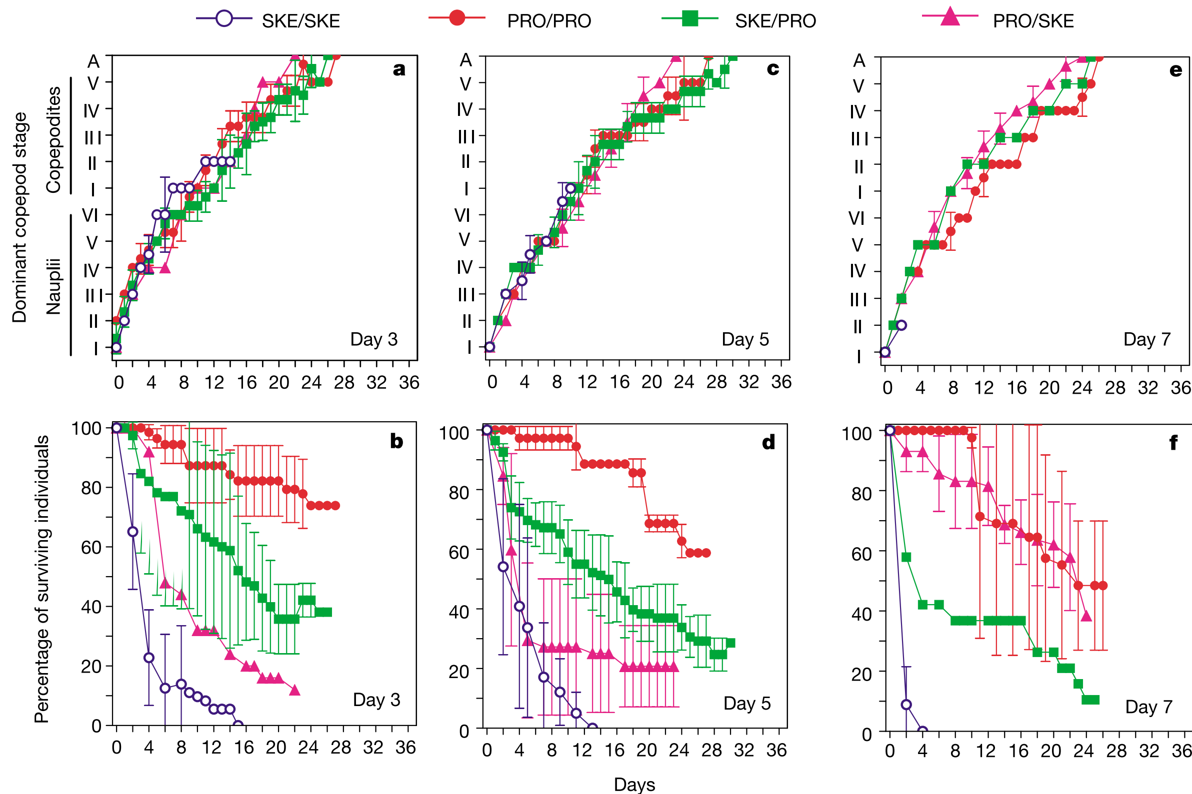

Figure 1 Effects of combinations of maternal and neonate diets on development rates (**a**, **c**, **e**) and percentage survivorship (**b**, **d**, **f**) of *C. helgolandicus* larvae. SKE/SKE and PRO/PRO are females and their larvae fed the diatom *S. costatum* (SKE) and the control dinoflagellate *P. minimum* (PRO), respectively. SKE/PRO indicates that females were fed SKE and their progeny fed PRO; the opposite is true for PRO/SKE. Experiments were run with eggs collected from females fed either of the diets for three (**a**, **b**), five (**c**, **d**) or seven (**e**–**f**) days. Values are mean ± s.d. for one to three replicates of 20 newly hatched nauplii. Bars for development curves are smaller than for percentage survivorship because of greater individual variability in death rates compared with growth rates for the same group of animals.

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depended on the days of exposure of the mother to the diatom diet (Fig. 1a–f). After three, five and seven days of feeding on SKE, development was arrested at copepodite stages CII, CI, and naupliar stage NII, respectively. Percentage survivorship was intermediate for the SKE/PRO and PRO/SKE diets and highest for PRO/PRO, although growth rates (reflected in development times to adulthood) were similar in all cases. Paired *t*-tests showed significant differences in daily survivorship but not in development rate (see Supplementary Information 1). The PRO diet resulted in decreasing survivorship with time (from 80% to 50% after three and seven days, respectively), suggesting a lack of some unknown essential nutrient(s) or other factor(s).

Nauplii collected from wild *C. helgolandicus* females during a diatom bloom from February to May 2003, dominated by SKE, *Stephanodiscus* sp. and *Chaetoceros* spp., did not survive to adulthood when reared on a diet of SKE (Fig. 2). Individuals died at or before the CII stage, except on the 13 and 27 March, when some nauplii (<20%) reached adulthood. Survivorship improved on the control diet PRO, but few reached adulthood except after late April.

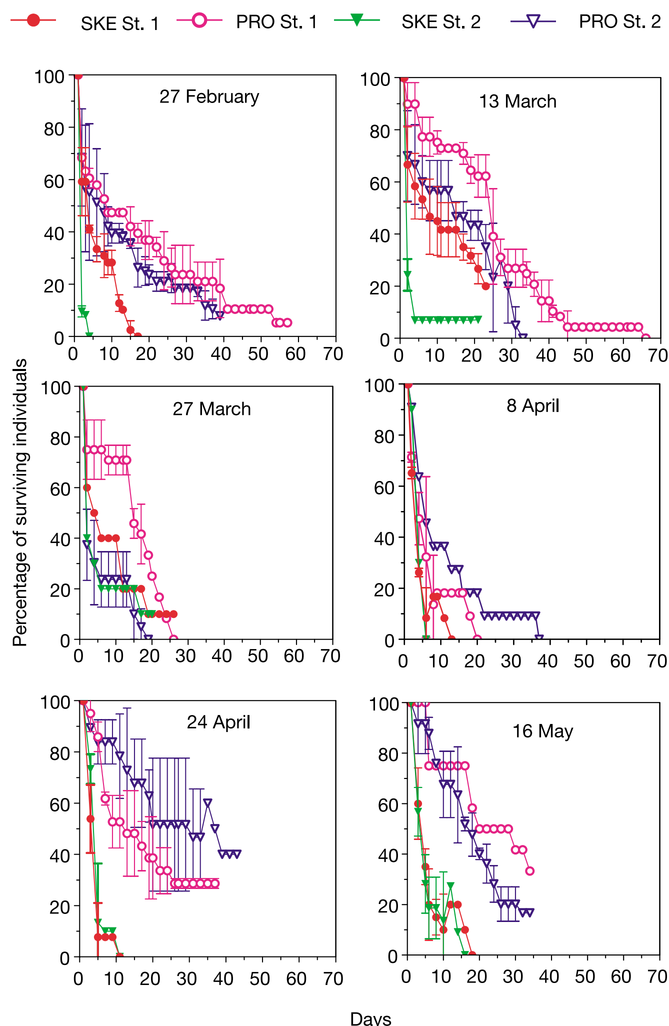

Figure 2 Percentage survivorship of *C. helgolandicus* nauplii spawned from wild females during the winter/spring bloom of 2003 in the North Adriatic Sea, and successively reared on the diatom *S. costatum* (SKE) or the dinoflagellate *P. minimum* (PRO). Values are mean ± s.d. for one to three replicates of 20 newly hatched nauplii. Experiments were run as in Fig. 1, on six sampling occasions and two different stations (St. 1 and St. 2). Adulthood was reached on only two occasions with SKE, with <20% survivorship, and on only three occasions with PRO, with highest survivorship (<40%) after mid-April.

Percentage egg viability during the bloom ranged from 16.8 ± 7.1 to 39.7 ± 7.5 (mean ± s.d.), similar to earlier results[11]. Thus, maternal diatom diets in the field negatively affect not only egg hatching success, but also the development of nauplii that do hatch.

Mothers fed on SKE produced nauplii with strong teratogenic (congenital) birth defects. First described for *C. helgolandicus* reared on a diatom diet (*Phaeodactylum tricornutum*)[14], the new experiments show that the reduction in egg viability (Fig. 3a) is accompanied by a concomitant rise in the number of hatched teratogenic nauplii (Fig. 3b). By day six, 45–65% of the hatched nauplii were malformed. Abnormal nauplii had asymmetrical bodies and malformed or reduced numbers of appendages (Fig. 3c, e, g). Some died within a few hours after hatching, whereas others died about one day later, at naupliar stage NII, because they were unable to swim or feed properly. Cell death through apoptosis had occurred in many tissues of the body, especially in appendages with strong structural malformations, and after nine days most nauplii were strongly deformed (Fig. 3d, f, h).

A diet of benthic diatoms can cause similar anatomical malformations in larvae of polychaetes and echinoderms[15]. The compounds responsible for these effects are short-chain α,β,γ,δ-unsaturated aldehydes that arrest embryonic development in copepods and sea urchins, and have antiproliferative and apoptotic effects on human carcinoma cells[11]. To test whether these aldehydes also arrested larval development in copepods, mothers were fed a culture of PRO to which concentrations of 0.5, 1.0 and 1.5 $\mu g\,ml^{-1}$ of the diatom-derived aldehyde 2-*trans*, 4-*trans* decadienal (DD) were added. Nauplii were then successively raised on SKE (Fig. 4). DD was chosen as a model aldehyde because its toxic properties are currently being tested on various animal cells[16,17,18]. The toxicity of DD and similar aldehydes more common in SKE[19] are all related to a reactive Michael acceptor structural element that is characteristic of different fatty-acid-derived diatom metabolites[3,20,21]. Because copepods do not drink[22], and the chitinous exoskeleton is impervious to aldehydes[18,23], DD can enter the animals only by adsorption on ingested PRO cells.

The results obtained at 1.5 $\mu g\,ml^{-1}$ were similar to those of feeding experiments (Fig. 1, SKE/SKE after three and five days), with nauplii dying at copepodite stage CI. At lower concentrations, some nauplii reached adulthood, but mortality was extremely high during the entire developmental phase. We calculated that females had ingested about 100 pg decadienal daily at 1.5 $\mu g\,ml^{-1}$, which is even below the range animals will encounter in the field, considering aldehyde production of about 30 fg per cell in wounded SKE (T.W., unpublished results) and ingestion rates of about 1,000 diatom cells per hour[24].

Our findings bring new insights to the century-long debate on the role of the spring diatom bloom in pelagic food chains leading to commercial fish stocks. Larval growth rates of copepods on pure diatom diets indicate sufficient food quality with no need for supplementation. Besides, a feeding experiment in which two strains of the cosmopolitan, bloom-forming diatom *Thalassiosira rotula*, one lacking the fatty-acid-cleaving enzyme, were fed to copepods clearly showed that only aldehyde production impairs recruitment[20]. A similar finding has been reported for an aldehyde-free strain of SKE fed to benthic animals[25]. Because reproductive success depends on the amount of aldehydes ingested, individual grazers with the least predilection for aldehyde-producing diatoms will be selected for. This would explain the hatching variability observed in blooms from different areas[12]. Indeed, copepods prefer motile protists (ciliates, dinoflagellates) over diatoms[26], so a mixed diet also serves to dilute the toxin[24]. Selective copepod feeding on protistan grazers of the diatoms further facilitates biomass build-up of blooms. The ungrazed biomass eventually sinks out, with significant consequences for ocean ecology and biogeochemistry.

In contrast to benthic and terrestrial realms, the study of grazer defences in the plankton is in its infancy[27]. Diatom frustules provide

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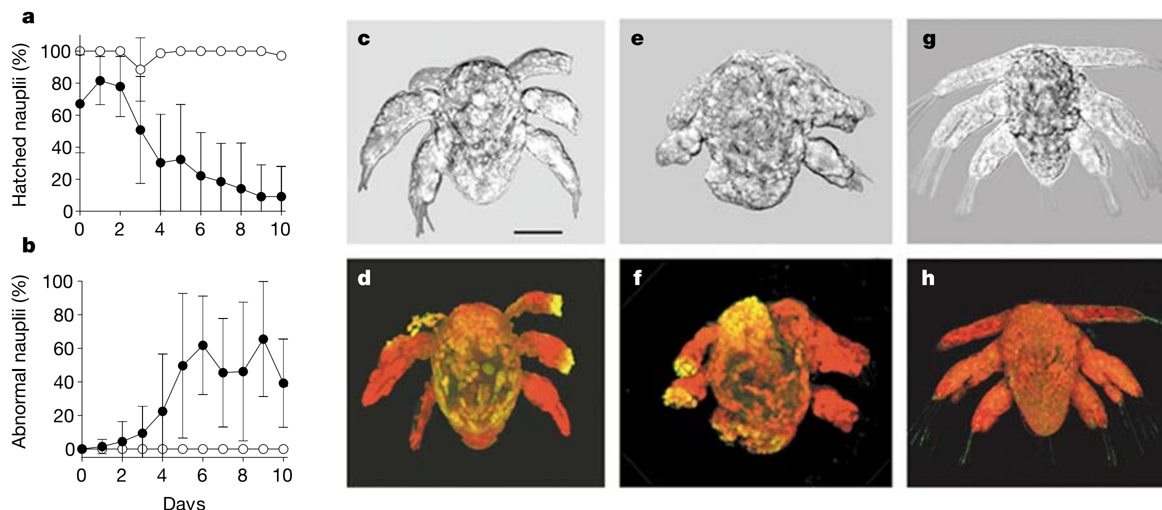

Figure 3 Effects of diet on *C. helgolandicus* offspring fitness. **a**, After ten days of feeding, the viability of eggs spawned by *C. helgolandicus* females fed the diatom *S. costatum* SKE (filled circles) dropped to <20% compared with >95% with the control dinoflagellate *P. minimum* PRO (open circles). **b**, After five days of feeding on SKE, 45–65% of the hatched nauplii were abnormal. **c**, **d**, Such nauplii had deformed limbs that were positive for TUNEL staining (yellow, **d**) specific for apoptosis. **e**, **f**, After nine days of feeding on SKE, the degree of teratogenesis increased and nauplii were strongly deformed. **g**, **h**, Nauplii generated from females fed the control PRO diet appeared normal and stained negatively with TUNEL (**h**), indicating that nuclei were not apoptotic. Scale bar, 90 μm.

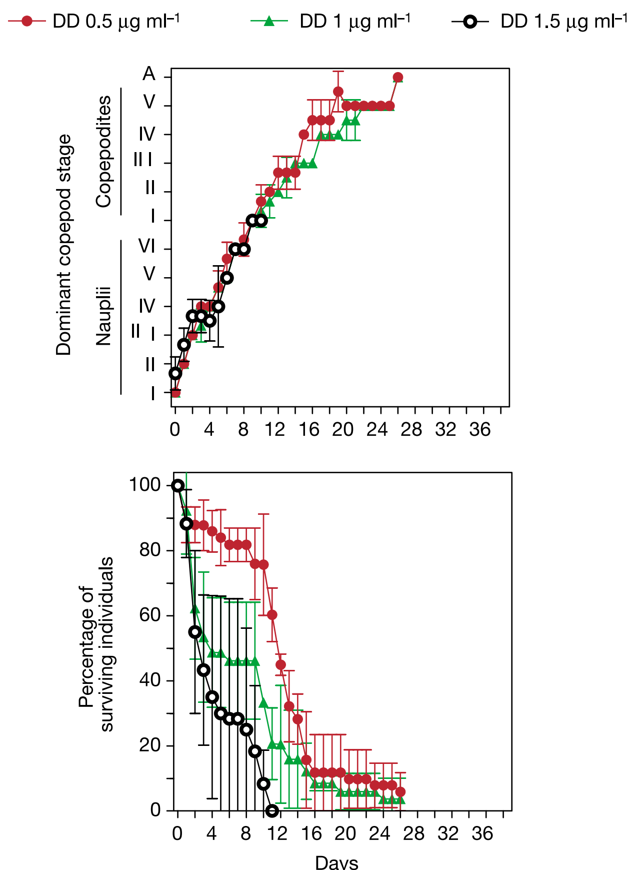

Figure 4 The effects of the aldehyde DD on development and percentage survivorship of *C. helgolandicus*. Females were fed the control dinoflagellate *P. minimum* (PRO) for three days, but with the addition of 0.5, 1.0 and 1.5 $\mu g\, ml^{-1}$ of decadienal. Nauplii were then raised on the diatom *S. costatum* (SKE). At lower concentrations, nauplii reached adulthood but survivorship was low. At the highest concentration, none of the nauplii reached adulthood and survivorship was only to copepodite stage CI.

mechanical protection against some classes of grazers[28], but the effect of chemical defences based on teratogens will be more subtle. Apparently, the success of bloom-forming species lies in pairing metabolically 'cheap' mechanical and chemical defences with fast growth rates. Our findings provide a plausible mechanism for the apparent poor timing between bloom development and the arrival of the bulk of the overwintering copepod stock. Because not all bloom-forming species produce aldehydes, competition between defended and innocuous species could explain the complex oscillations[29] between diatom and copepod populations observed over decadal scales[7]. An evolutionary arms race operating in the plankton[30] could well be a major force shaping the annually recurring, yet unexplained patterns of plankton species succession. □

Methods

Rearing experiments

Female *C. helgolandicus* copepods, collected offshore of Roscoff (France) and Chioggia (Italy), were pre-conditioned for 24 h in crystallizing dishes filled with 100 ml of 0.22 μm filtered sea water enriched with the dinoflagellate *P. minimum* (PRO) at a final concentration of 8.6×10^3 cells per ml, and kept at 17 °C and on a 12 h light/12 h dark cycle. Females were then divided into two groups and transferred to new containers with either PRO (control) at the same concentration, or the diatom *S. costatum* (SKE) at 7.3×10^4 cells per ml so that final algal concentrations were equivalent in terms of carbon (PRO = 177.1 pg per cell and SKE = 20.7 pg per cell). Methods for cultivation of algae are reported elsewhere[24]. Females were transferred to new culture media daily. After three, five and seven days of feeding, one to three replicates of 20 N1, from females fed either PRO or SKE, were placed in 300-ml jars with 0.22 μm filtered sea water containing, in turn, either PRO or SKE, so that four combinations of diets were tested: those in which females and offspring both received PRO (control) or both received SKE; and those in which females received either PRO or SKE, and their offspring the other diet. Juveniles were collected daily, counted and checked under the microscope to determine the stage of development, and transferred to jars with fresh medium. A tally was kept of the most abundant larval stage and percentage of surviving individuals, to calculate development and survival curves. Rearing experiments were also conducted on nauplii collected from wild females during the winter/spring diatom bloom of 2003 in the North Adriatic Sea. Copepods were collected at two stations representative of the coastal (station 2), and offshore (station 1) areas, separated by a haline front[6]. Immediately hatched N1 were collected and transferred to SKE and PRO diets; developmental stage was determined as above. Rearing experiments testing the effects of DD (Sigma) were conducted using the same procedures as above. DD was dissolved in methanol to obtain a stock solution of 0.3 $mg\, ml^{-1}$. Females were fed PRO cultures to which 0.5, 1.0 and 1.5 $\mu g\, ml^{-1}$ of DD were added for three days; the methanol solvent added had no negative effect on copepods or their eggs, up to 1% in the final solution. Nauplii collected on day four were then reared on a diet of SKE.

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Determining the amount of DD ingested by copepods

Samples of 40 ml of PRO (8.0×10^4 cells per ml) were incubated in sea water with 1.5 $\mu g\, ml^{-1}$ of DD. After different incubation times, the cells were filtered onto GF/C filter (21 mm, Whatman) under reduced pressure until dry. The filter was rinsed with 1 ml 25 mM pentafluorobenzylhydroxylamine in 100 mM Tris-HCl, pH 7.0. After addition of benzaldehyde (5 µl of a 1 mM solution in methanol) as an internal standard, the cell suspension was sonicated in an ice bath with pulses of a B. Braun 1000 l Sonicator for 1 min. Afterwards, the sample was incubated at room temperature for 30 min. Extraction was performed with hexane[31]. Each treatment was replicated three times. Triplicate controls, consisting of sea water and DD without PRO, were conducted to determine the filter adsorption of DD. Detection was performed with gas chromatography/mass spectrometry (GC/MS) (GC Q; equipped with a 30 m RTX-200 column, 0.25 mm internal diameter, 0.25 µm film thickness). The analyses were performed by negative ion chemical ionization electron-capture mass spectrometry with methane as the reagent gas. For quantification of DD, the ion at m/z 327 $[M-HF]^{\cdot-}$ was chosen. A calibration curve shows linearity ($r^2 > 0.98$) in the measurement range (see Supplementary Information 2).

Assessment of Apoptosis

Egg hatching success and number of teratogenic nauplii were also monitored daily with the two diets. Procedures to determine egg-hatching success are described elsewhere[24]. Apoptosis in teratogenic nauplii was verified using TdT-mediated dUTP nick end labelling (TUNEL) (Roche Diagnostics). *C. helgolandicus* nauplii were fixed overnight in 4% paraformaldehyde and 0.2 M NaCl in PBS, pH 7.4, rinsed in PBS, and frozen in liquid nitrogen to fracture the carapace. Samples were incubated for 24 h in 1 unit ml^{-1} chitinase enzyme (Sigma) at 25 °C, and rendered permeable according to the TUNEL manufacturer's instructions. They were then incubated for 90 min at 37 °C in TUNEL reaction mix and for 30 min in 0.5 $\mu g\, ml^{-1}$ propidium iodide at room temperature. Nauplii were observed with a confocal laser-scanning microscope, Zeiss LSM-410, in which TUNEL-positive areas appear yellow because of the superimposition of the green fluorescence of TUNEL and the red fluorescence of propidium iodide. Complementary tests with a mammalian cell line (A1 mes c-myc cells), generated from mouse mesencephalon primary cultures, suggest that DD is potentially a neutral compound for somatic but not for embryonic development, affecting undifferentiated proliferating rather than differentiated non-proliferating cells (see Supplementary Information 3).

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

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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 (0–2×10^6 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}

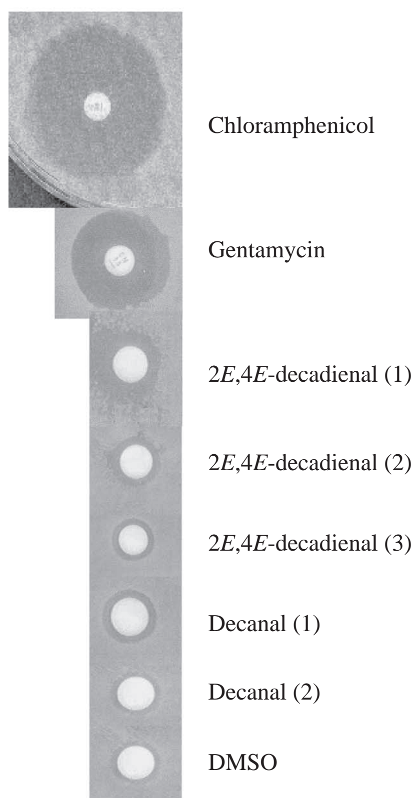


Fig. 1. *Vibrio splendidus* (bacterium). Effects of the aldehydes decanal (1=66 μg disk^{-1}, 2=6.6 μg disk^{-1}) and 2*E*,4*E*-decadienal (1=33.3 μg disk^{-1}, 2=6.6 μg disk^{-1}, 3=0.66 μg disk^{-1}) on cell proliferation, shown by the growth inhibition zone around the disk at different dilutions.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

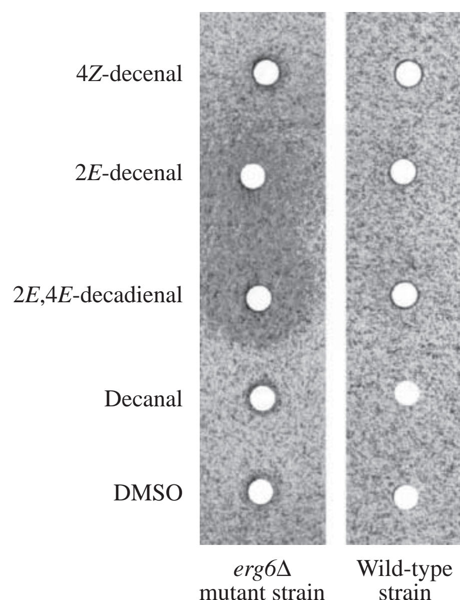


Fig. 2. *Saccharomyces cerevisiae* (fungus). *erg6Δ* cells are insensitive to decanal and 4*Z*-decenal but are highly sensitive to 2*E*,4*E*-decadienal and 2*E*-decenal, as indicated by the growth inhibition halo around the filter where these molecules were spotted. Concentration of each aldehyde tested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

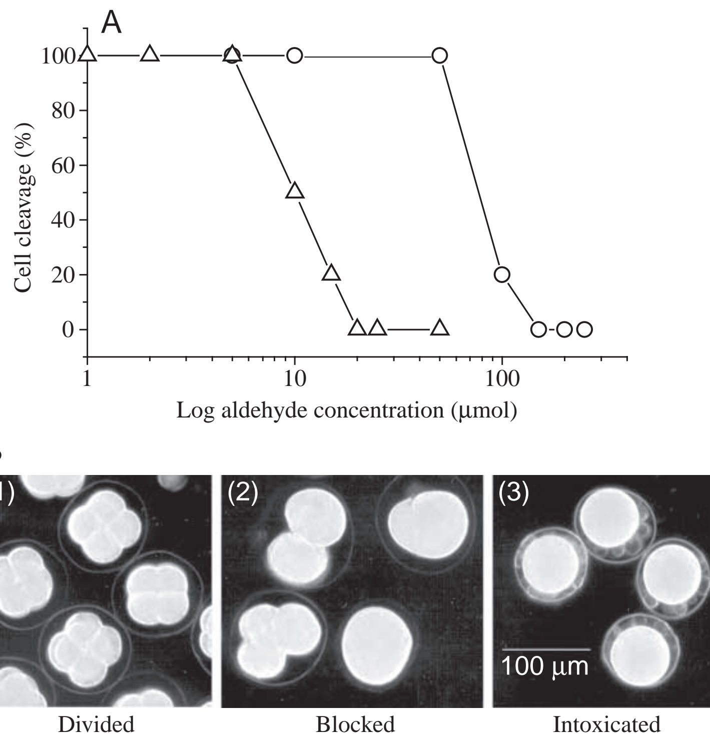


Fig. 3. *Sphaerechinus granularis* (echinoderm). (A) Dose-dependent effects of decanal (circles) and 2*E*,4*E*-decadienal (triangles) on cell division during the early embryogenic phase (four blastomeres) in 2.5 h-old embryos. Values are means of three replicate measurements. Standard error (<3% of the 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

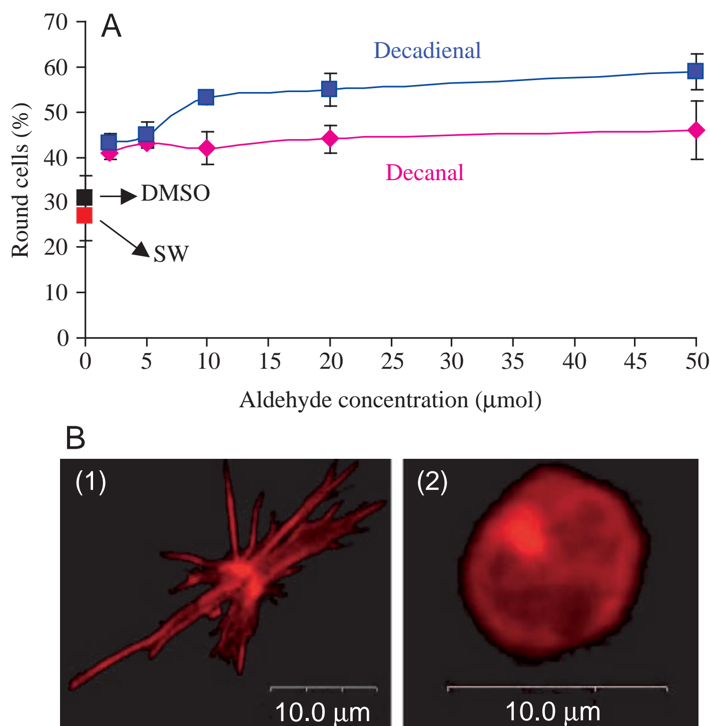


Fig. 4. *Crassostrea gigas* (mollusc). (A) Dose-dependent response of oyster haemocytes incubated in seawater (SW, controls), DMSO (solvent control), 2*E*,4*E*-decadienal and decanal. Values (mean ± S.D.) are the estimates of the proportions of abnormal, round cells reflecting the impact of these treatments 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).

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

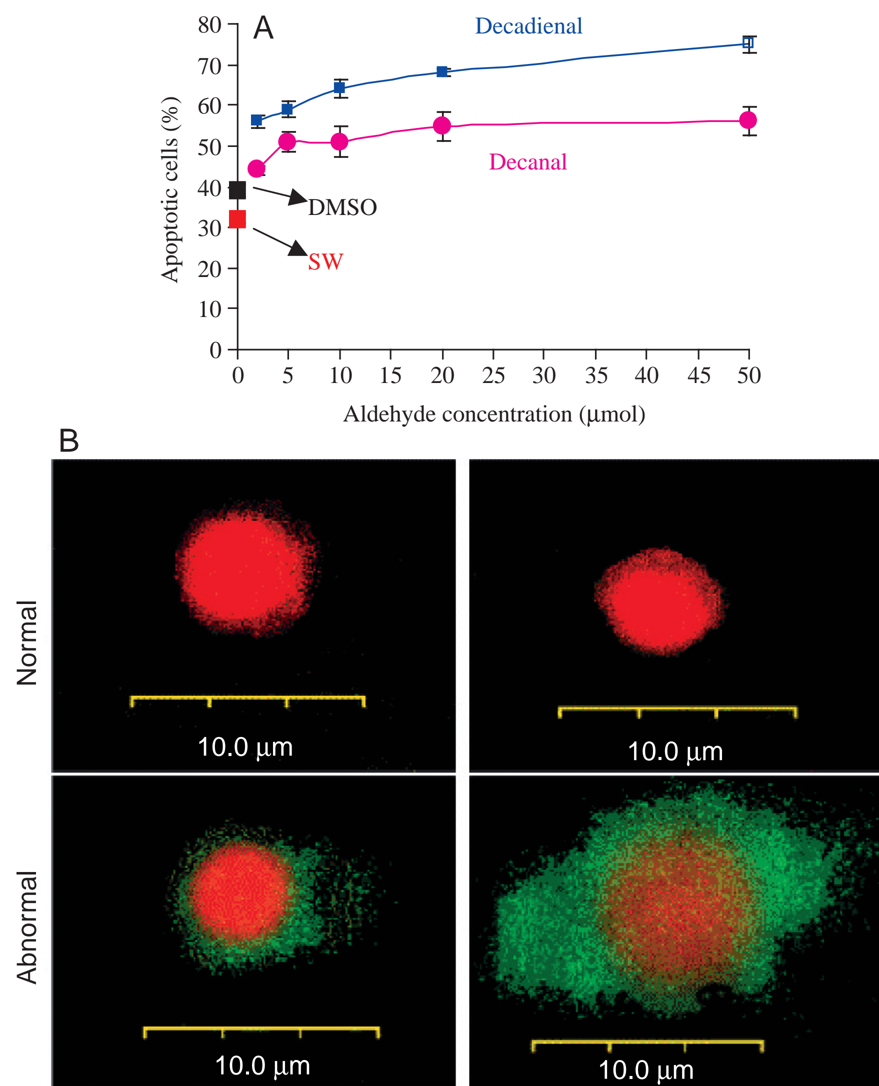


Fig. 5. *Crassostrea gigas* (mollusc). (A) Dose-dependent response of oyster haemocytes incubated in seawater (SW, controls), DMSO (solvent control), 2*E*,4*E*-decadienal and decanal. Values (mean ± S.D.) are the proportions of abnormal, apoptotic cell degradations reflecting the noxious impact of the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

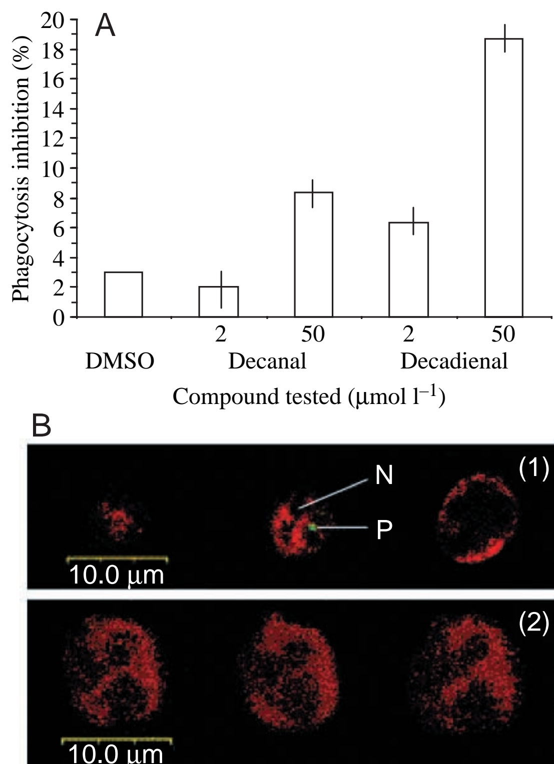


Fig. 6. *Crassostrea gigas* (mollusc). (A) Response of oyster haemocytes incubated in 2*E*,4*E*-decadienal or decanal (at concentrations of 2 and 50 µmol l^{-1}) and DMSO (solvent control). Values (mean ± S.E.M.) are given for three replicate tests, showing the proportions of blood cells presenting phagocytosis 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.

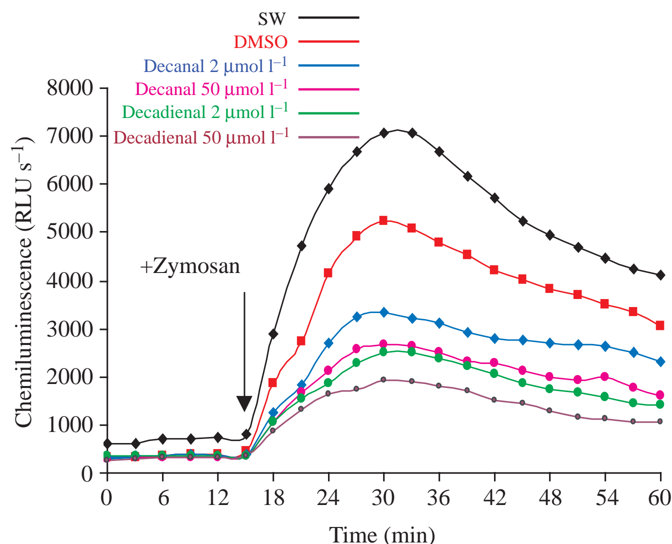


Fig. 7. *Crassostrea gigas* (mollusc). Concentration-dependent inhibition of the luminol-dependent chemiluminescence response of oyster haemocytes from a common pool of cells (10^6 cells ml^{-1}) by aldehydes (at concentrations of 2 and 50 µmol l^{-1}). For each treatment, values are the results of triplicate 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

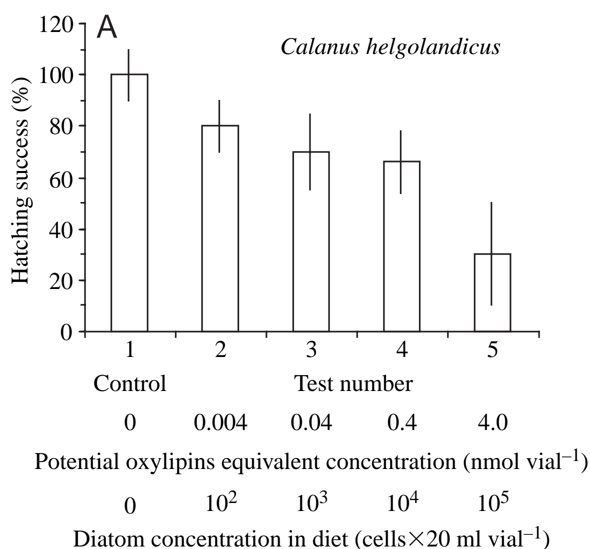
Results reported here show that diatom-derived α,β,γ,δ-unsaturated aldehydes induced, *in vitro* and *in vivo*, antiproliferative activity in a broad range of organisms. The saturated aldehyde decanal had no detectable or only minor inhibitory effects. According to Adolph et al. (2003), the antiproliferative 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



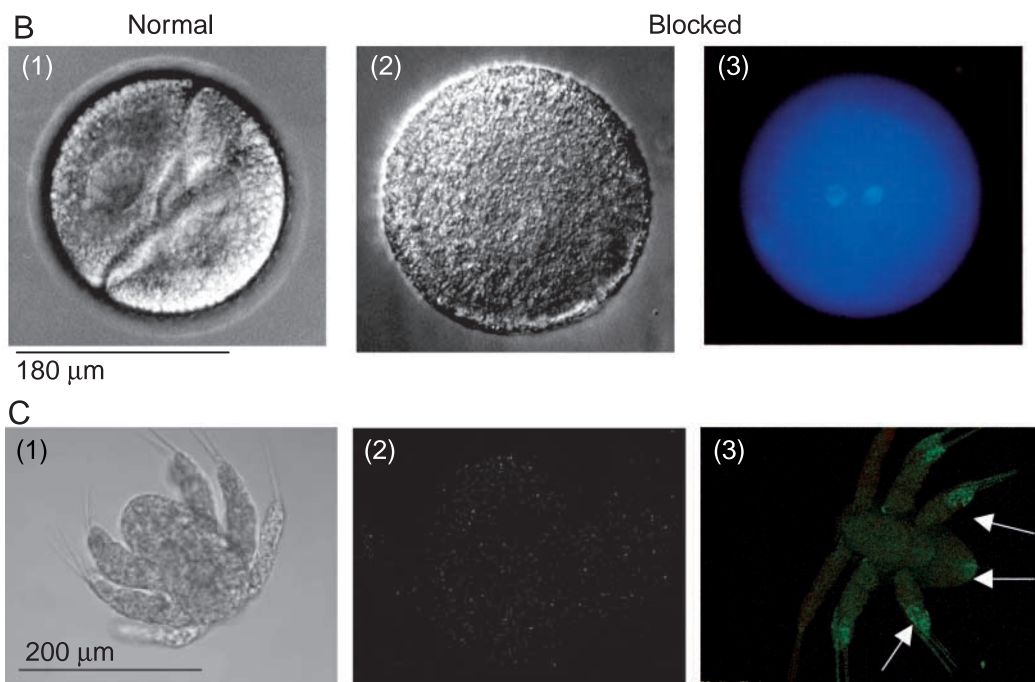


Fig. 8. *Calanus helgolandicus* (crustacean). (A) Post-ingestion effect of the noxious diatom *Thalassiosira rotula*, provided to spawning females. Dose-dependent values (mean ± S.D.) of the hatching success are the results of triplicate observations for each diet treatment in batch samples of 30–100 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, neuro-transmitters, 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 $\alpha,\beta,\gamma,\delta$-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.

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Life-history responses of *Daphnia pulicaria* to diets containing freshwater diatoms: Effects of nutritional quality versus polyunsaturated aldehydes

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Abstract

Like marine diatoms, some freshwater diatoms produce $\alpha,\beta,\gamma,\delta$-polyunsaturated aldehydes (PUA) when damaged. Some of these oxylipins are suspected of impairing egg viability in marine copepods. To determine whether these compounds also play a role in influencing the trophic interactions in freshwater environments, we measured growth and reproduction of the cladoceran *Daphnia pulicaria* in response to diets composed of seven diatoms differing in PUA production. The juvenile growth rate of *Daphnia* varied with the diatom species, but was not related to oxylipin production. Egg hatching success was nearly 100% in all clutches for all diets, except with a diet of the decadienal producing *Fragilaria* sp., where it decreased dramatically in clutches 5–7. In vitro tests of egg hatching in the presence of PUA showed a dose-dependent inhibition for decadienal. Population parameters (i.e., life-time fecundity and instantaneous rate of population growth) were not affected by PUA, as the contribution of late clutches to them was negligible. Consequently, the wound-activated production of PUA by diatoms cannot be regarded as a defensive mechanism against *Daphnia* population recruitment.

Marine diatoms have been considered a main path of the energy flow from primary producers to copepods (Ryther 1969), but their central role is now under debate. Many copepods, in fact, are omnivorous and prefer protists (Kleppel 1993), and various diatoms have been found to have deleterious effects on copepod reproduction in the laboratory and in the field (Ban et al. 1997; Miralto et al. 1999). As freshwater diatoms can represent a considerable portion of phytoplankton biomass in lakes during seasonal succession (Sommer et al. 1986), they may be frequently included in the *Daphnia* diet. There is evidence that small single-celled diatom species like *Stephanodiscus hantzschii* are high-quality food for daphnids and provide high somatic and population growth rates and fecundity (Infante and Litt 1985; Lundstedt and Brett 1991), while the nutritional value of larger colonial species like *Asterionella* and the genus *Fragilaria* is lower (Infante and Litt 1985).

In recent years, copepods fed widespread bloom-forming diatoms (e.g., *Thalassiosira rotula* and *Skeletonema costatum*) were found to have low egg-hatching success and erratic embryonic development (for a review, see Ianora et al. 2003). These effects were correlated with the occurrence of a group of structurally diverse $\alpha,\beta,\gamma,\delta$-polyunsaturated aldehydes (PUA) produced by mechanically damaged diatom cells (Miralto et al. 1999; Pohnert 2000). PUA belong to the group of oxylipins, compounds derived from the oxidative transformation of polyunsaturated fatty acids (Pohnert 2000), and their production has been interpreted as a diatom defense affecting copepod recruitment into the next grazer generation (Ianora et al. 2004). Among the PUA, 2,4-decadienal as well as other shorter chain-length volatile aldehydes and oxo-acids, all bearing the same structural $\alpha,\beta,\gamma,\delta$-polyunsaturated aldehyde motive, have been found to impair copepod hatching success and cleavage of sea urchin eggs in vitro (d'Ippolito et al. 2002*a,b*; Pohnert et al. 2002; Adolph et al. 2003).

It is interesting that the same reactive oxylipins have been detected in freshwater diatoms. They were reported from epilithic diatom biofilms (Jüttner and Durst 1997) and in lake water during the senescent phase of phytoplankton blooms (Watson et al. 2001). In particular, the freshwater diatoms *Fragilaria* sp. and *Melosira varians* produced the volatile aldehydes 2,4-heptadienal, 2,4-octadienal, and 2,4-decadien-

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al (Wendel and Jüttner 1996). *Asterionella formosa* and *Gomphonema parvulum* produce the closely related polyunsaturated oxo-acids, 12-oxo-dodeca-5,8,10-trienoic acid (12-ODTE) and 9-oxo-nona-5,7-dienoic acid (9-ONDE), respectively. Similar lipoxygenase/hydroperoxide lyase reactions as described for marine diatoms are involved in the biosynthesis of these metabolites in freshwater diatoms (for a review, see Pohnert and Boland 2002).

Despite the known production of PUA in freshwater diatoms, their effect on freshwater grazers has rarely been studied. Short-term feeding experiments (Carotenuto and Lampert 2004) rendered the role of PUA as feeding deterrents for *Daphnia* unlikely, but there is still the possibility of long-term effects on growth and reproduction, as in marine copepods. For example, arrested egg development and egg abortion have been observed in field populations of *Daphnia* (Threlkeld 1979; Boersma and Vijverberg 1995) and have been linked to food composition but not to particular species of diatoms. We tested the hypothesis that *Daphnia* react like marine copepods in their responses to diatom diets. We compared the effects of seven diatom species varying in colonial shape and PUA production on the growth and reproduction of *Daphnia.* Concentrating on parameters that determine the fitness of *Daphnia,* we studied the question of whether the wound-activated production of particular oyxlipins by diatoms can be regarded as a defense mechanism against *Daphnia* grazing pressure.

Materials and Methods

Algal culture and preparation of food suspensions—Seven nonaxenic diatom species (*S. hantzschii, Stepanodiscus minutulus, Cyclotella meneghiniana, A. formosa, Fragilaria* sp., *Fragilaria capucina, G. parvulum*) were cultivated in 1-liter conical flasks in modified WC medium (Guillard and Lorenzen 1972) in a growth chamber at 16°C under a 16 : 8 light : dark (LD) cycle. The nonaxenic green alga *Scenedesmus obliquus* was grown in a chemostat in modified CHU12 medium (Müller 1972) at 20°C and continuous light. Properties of six of the strains are presented in Carotenuto and Lampert (2004). *G. parvulum* has similar cell size as the other diatoms and tends to form aggregates.

For use in life-history experiments, stock algal cultures were diluted with membrane-filtered water from a nearby mesotrophic lake (Schöhsee) to a final concentration of 1 mg carbon L^{-1}, which is above the incipient limiting concentration for *Daphnia* (Lampert 1987). The density of the stock algae was estimated by measuring the light extinction (800 nm) in a photometer. The final algal concentration was then adjusted using a separate calibration curve (extinction vs. particulate carbon) for each algal species.

Chemical analysis of diatoms—Screening of exponential growth-phase cultures of the seven diatom species for volatile aldehyde production was performed with modifications based on an established procedure (Pohnert et al. 2002). The algal cultures were gently filtered onto GF/C filters (Whatman). The filters were placed in 5-ml glass vials and the pellet was resuspended in 0.5 ml medium to a final concentration of 10^6–10^7 cells ml^{-1}. Saturated NaCl solution (0.5 ml) was added to damage cells by osmotic pressure. The samples were then sonicated and extracted with solid-phase microextraction as described by Pohnert et al. (2002). Identification of PUA was performed by comparison with synthetic (Pohnert 2000; Adolph et al. 2003) or commercially available reference compounds. For quantification of volatile PUA, samples were prepared as described above, derivatized with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride, and extracted with hexane according to Ianora et al. (2004). The nonvolatile acidic aldehydes 12-ODTE and 9-ONDE were detected and quantified by reversed phase high-performance liquid chromatography-mass spectroscopy according to Pohnert (2000). Sample preparation included filtration and damaging of the cells by sonication, methanol-precipitation of proteins, and centrifugation.

Life-history trait experiments—Stocks of *D. pulicaria* Forbes were maintained in a temperature-controlled room at 19°C ± 1°C and continuous dim light. They were kept in 1.5-liter jars with filtered Schöhsee lake water and fed *S. obliquus* three times a week. Juveniles of different ages ($n = 90$) were collected from the stocks and raised individually in 200-ml jars with lake water and 1 mg C L^{-1} of *Scenedesmus* until they produced their third clutch of eggs. Offspring from the third brood were used to initiate the growth experiments in the different food conditions. We performed two series of experiments: one to determine growth rate of individuals during the juvenile phase, a good proxy of fitness, the second to calculate effects on population parameters by measuring survival, fecundity, and hatching success. To start experiment 1, a random subsample of ten 24-h-old neonates was dried overnight at 60°C, cooled in a desiccator, and weighed to the nearest 0.1 μg to determine their initial dry mass (W_0). The remaining individuals were randomly distributed among the different experimental treatments, consisting either of monoalgal diatom food and *S. obliquus* or of mixtures (1 : 1 in terms of carbon) of the individual diatoms and *S. obliquus* at always 1 mg C L^{-1}. Six neonates per treatment were raised individually in 100-ml jars, fed daily, and transferred to clean jars with new medium every second day. When they reached maturity and deposited the first clutch of egg into the brood chamber, they were sacrificed, dried, and weighed (W_1). The juvenile growth rate g_j (d^{-1}) was calculated according to the equation $g_j = (\ln W_1 - \ln W_0)/t$ (Lampert and Trubetskova 1996), where t is the duration of the experiment (days). W_1 always included the first clutch of eggs in the brood chamber, hence g_j represents the sum of somatic growth and reproduction of the juveniles. The number of eggs produced by each individual in the first brood was also recorded.

In the second series of experiments, a group of third-clutch neonates of *D. pulicaria* was raised on a *Scenedesmus* diet (1 mg C L^{-1}) until the daphniids deposited the first clutch of eggs into the brood pouch. These young adults were then subjected to the same temperature and food conditions as in the first series, and their egg production and hatching success was monitored over the next seven broods. For each food treatment, 16 females were incubated individually in 200-ml jars filled with the appropriate food suspension (1 mg C L^{-1} in filtered lake water), fed daily, and trans-

Table 1. Means (±1 SD, n=6) of the juvenile growth rate (g_j) of *D. pulicaria* fed seven diatom species and *Scenedesmus* either as pure diet or in mixtures of one diatom and *Scenedesmus* (1:1 in terms of carbon).

	Diet type	
	Monoalgal	Mixed
Food species	g_j (d^{-1})	g_j (d^{-1})
S. obliquus	0.578 (0.151)	—
S. minutulus	0.661 (0.117)	0.709 (0.031)
A. formosa	0.520 (0.095)	0.651 (0.036)
S. hantzschii	0.476 (0.078)	0.673 (0.038)
C. meneghiniana	0.442 (0.085)	0.659 (0.024)
G. parvulum	0.433 (0.038)	0.684 (0.044)
F. capucina	0.357 (0.028)	0.649 (0.022)
Fragilaria sp.	0.298 (0.083)	0.652 (0.023)

ferred to fresh medium every other day. The number of eggs in the brood pouch of each female was counted under a stereomicroscope each time the medium was changed and the number of live neonates and nonviable eggs was recorded after pouring the contents of the jars through a 100-μm mesh. In addition to the number of viable offspring, the reproduction experiment yielded the numbers of survivors of the original cohort over time and the times of neonate release for the successive broods. These data were used to calculate age-specific survival probability (l_x) and age-specific fecundity (m_x). The instantaneous rate of increase (r) was then calculated for every individual using the Euler equation (Stearns 1992).

In vitro assay with Daphnia embryos—D. pulicaria juveniles from the stock cultures were individually reared in 200-ml jars with 1 mg C L^{-1} *Scenedesmus.* As soon as they released their second clutch of neonates, they were checked every 30 min to detect the deposition of another clutch into the brood pouch, which was considered the zero time of egg development. Only eggs from the third to the fifth broods were used to initiate an in vitro experiment. Six hours after deposition, the eggs were removed from the brood pouch under a dissecting microscope and washed twice with 0.2-μm filtered lake water. To determine the in vitro influence of PUA on the development of embryos, one representative of the oxo-acids (12-ODTE) and one from the volatile aldehydes (2,4-decadienal) were tested. Eggs isolated from 5–6 females were pooled and a batch of 10 was randomly transferred into each depression of a six-well tissue culture plate that contained 5 ml of freshwater with 0.5–3.0 μg ml^{-1} of 2,4-decadienal (SIGMA, Aldrich) in 1.2–7.5 μl ml^{-1} methanol or 1–4 μg ml^{-1} of synthetic 12-ODTE (Pohnert 2000) in water. The appropriate amount of methanol was used as control in the decadienal treatment, whereas freshly filtered water was used in the 12-ODTE treatment. Embryos were maintained in a temperature-controlled chamber at 20°C and 16:8 LD cycle. Five replicates were performed for each treatment. Egg development was monitored until the embryos shed the external membrane (less than 24 h after their incubation), which was considered successful hatching.

Table 2. Results of GLM ANOVAs on life history variables of *D. pulicaria* fed seven species of diatoms or mixtures of one diatom and *Scenedesmus* (1:1 in terms of carbon) with estimation of the main effects of diatom species (spec) and presence of *Scenedesmus* (mix) and interactions between these. Life-history variables juvenile growth rate (g_j) and number of eggs in the first clutch (eggs) were measured in the first experimental series. The population average cumulative number of eggs in seven clutches (R_o) and the instantaneous rate of increase (r) result from the second experimental series.

Variable	Factor	Degrees of freedom	Mean square spectroscopy	F	p
g_j	Spec	6, 70	5.33	14.1	<0.001
	Mix	1, 70	0.94	251.4	<0.001
	Spec × mix	6, 70	3.05	8.1	<0.001
eggs	Spec	6, 70	8.01	2.0	0.078
	Mix	1, 70	146.67	36.6	<0.001
	Spec × mix	6, 70	1.90	0.5	0.826
R_o	Spec	6, 196	3032	2.5	0.025
	Mix	1, 196	33555	27.3	<0.001
	Spec × mix	6, 196	395.9	0.3	0.925
r	Spec	6, 196	0.014	8.0	<0.001
	Mix	1, 196	0.123	70.2	<0.001
	Spec × mix	6, 196	0.008	4.5	<0.001

Results and discussion

Among the diatoms tested, only three species produced detectable amounts of PUA. *A. formosa* and *G. parvulum* produced 12-ODTE and 9-ONDE, respectively, whereas *Fragilaria* sp. produced 2,4-heptadienal, 2,4-octadienal, and 2,4-decadienal. The other algae tested did not give any detectable PUA even if highly concentrated samples (10^7 cells ml^{-1}) were analyzed. The detection limit for the volatile aldehydes was around 0.1 fmol per cell, depending on the cell size.

All *D. pulicaria* neonates reached maturity both with monoalgal and mixed diets. Algal species had a significant effect on juvenile growth rate (g_j) with monoalgal food (Table 1), but not on the number of eggs in the first clutch (mean = 6.60, SD = 2.29, n = 48). The highest g_j was found for *S. minutulus* and *A. formosa,* not significantly different from the *Scenedesmu*s control. The two *Fragilaria* species resulted in the lowest g_j, but their effects on this parameter did not differ from each other. In general, part of the differences in growth rates with monoalgal food was caused by later egg laying of daphnids.

The diatom species effect on g_j was only marginally significant if they were mixed with *S. obliquus.* All mixed diets resulted in higher g_j than pure *Scenedesmus.* For comparison of the effects of pure and mixed diets, we excluded *Scenedesmus* from the pure algal data set and analyzed the data by two-way ANOVA (Table 2) with diatom species and presence or absence of *Scenedesmus* as main effects. Addition of *Scenedesmus* had significant effects on g_j and eggs in the first clutch. There were also significant interactions between species and *Scenedesmus* addition for g_j, indicating a different response to the diatom constituents of the diet. This interaction was not found for the eggs; hence, egg num-

Table 3. Life history parameters of *D. pulicaria* fed seven diatom species and *Scenedesmus* either as pure diet or in mixtures of one diatom and *Scenedesmus* (1 : 1 in terms of carbon). Data include seven broods. Survival denotes the fraction of females that finished the seventh brood.

	Diet type							
	Monoalgal				Mixed			
Species	n	Survival (%)	R_o	r (d^{-1})				
S. obliquus	16	18.8	24.1 (32.9)	0.197 (0.050)	—	—	—	—
S. hantzschii	13	46.2	47.0 (39.0)	0.246 (0.035)	9	77.8	80.9 (51.5)	0.281 (0.034)
S. minutulus	16	37.5	36.9 (31.7)	0.245 (0.045)	12	58.3	70.6 (54.2)	0.282 (0.034)
A. formosa	16	37.5	27.4 (25.3)	0.236 (0.037)	13	46.2	58.2 (48.4)	0.272 (0.048)
C. meneghiniana	16	31.3	35.1 (27.4)	0.228 (0.035)	16	43.8	52.3 (47.9)	0.267 (0.032)
G. parvulum	16	43.8	26.2 (20.0)	0.211 (0.039)	16	43.8	41.9 (37.0)	0.225 (0.058)
Fragilaria sp.	26	50.0	21.8 (13.0)	0.173 (0.024)	12	41.7	46.0 (34.2)	0.277 (0.074)
F. capucina	16	43.8	26.1 (22.7)	0.168 (0.039)	13	53.9	52.4 (40.1)	0.251 (0.043)

bers were improved by adding *Scenedesmus* to all diatom species (mean = 9.17, SD = 1.78, n = 42).

Not all *D. pulicaria* females survived until they released their seventh clutch of eggs. Surprisingly, *Daphnia* fed pure *Scenedesmus* had the lowest survival of all treatments (Table 3). Although overall the pure diatom diets seemed to result in somewhat lower survival (41.4 ± 6.3%) compared with the mixed diets (52.2% ± 12.8%), neither a two-sample t-test (t = 2.0, p = 0.078) nor a paired t-test (t = 2.18, p = 0.072) resulted in a significant difference between the groups.

Survival and age-specific fecundity determined the total number of eggs per female (R_o) produced during seven broods. Due to the varying lifetime of the individuals, R_o showed a large variability (Table 3). A two-way ANOVA after exclusion of the pure *Scenedesmus* treatment resulted in a marginally significant effect of diatom species, whereas the addition of *Scenedesmus* led to significantly higher lifetime reproduction in all treatments (Table 2).

The instantaneous population growth rate (r) was much less variable than R_o, showing a similar contrast between diatom diets as for g_j, with the lowest r-values for the two *Fragilaria* species and the highest for the two *Stephanodiscus* species (Table 3). Differences between species were much smaller for the mixed diets. The two-way ANOVA (Table 2) revealed significant effects of species, diet composition, and the interaction between them. Supplementation of the diatoms with *Scenedesmus* led to an increase of r, but the response varied for different combinations.

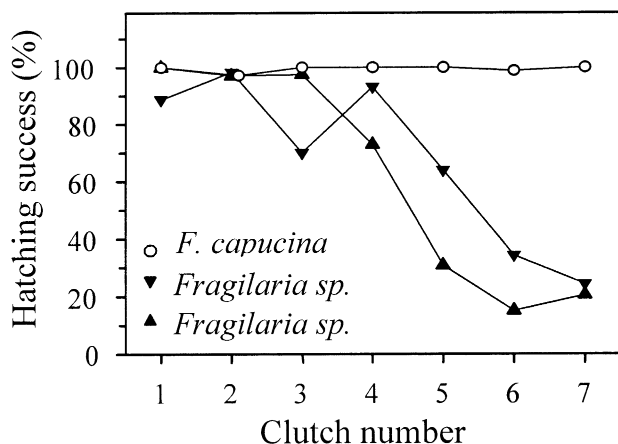

Fig. 1. Percentage of viable eggs in seven broods of *D. pulicaria* fed pure diets of *Fragilaria* sp. (two experiments) or *F. capucina.*

The mean hatching success in all treatments except pure *Fragilaria* sp. was always above 90%. There was no time trend and a typical example is presented as for the *F. capucina* diet in Fig. 1. With pure *Fragilaria* sp. as diet, egg viability dropped dramatically to values as low as 20% after the fourth clutch. This experiment was successfully repeated with very similar results (Fig. 1). Addition of *Scenedesmus* eliminated the effect completely.

D. pulicaria embryos incubated in water containing synthetic 12-ODTE at increasing concentrations (1–4 μg ml^{-1}) did not alter the normal pattern of development, and 100% of the embryos moulted successfully into the second embryonic stage, like the controls incubated in filtered lake water (Fig. 2). The egg membrane was cast off 14–16 h after the incubation, i.e., 20–22 h after deposition into the brood pouch of the female. In contrast, embryos incubated in 2,4-decadienal at concentrations from 0.5 to 3.0 μg ml^{-1} showed a dose-dependent impairment of development. Up to 1.5 μg ml^{-1}, about 80% developed through the first instar, which was consistent with the pure-methanol controls. Only 40% shed the first membrane at 2.5 μg ml^{-1}, and none of the embryos developed at 3.0 μg ml^{-1} (Fig. 2).

Our experiments covered two phases of the *Daphnia* life cycle, juvenile growth and the reproductive phase, both integrating metabolic processes like feeding, assimilation, and respiration. Both phases can be affected by nutritional inadequacy and toxic compounds of the diet. While an earlier study (Carotenuto and Lampert 2004) tested the short-term effects on *Daphnia* carbon uptake caused by algal morphology, digestibility, assimilation efficiency, and oxylipin production, this study looks at long-term effects on individuals and the population.

Besides the morphological differences of cells and colonies, the diatoms selected differ in the ability to produce PUA upon cell damage. Several species are nonproducers, whereas others are similar to well-investigated marine PUA

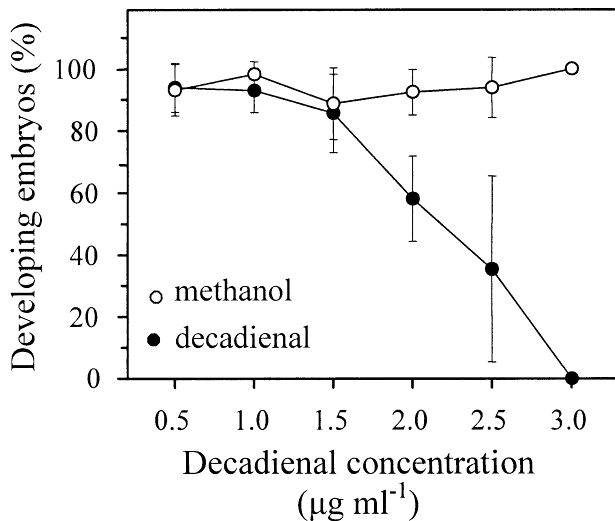

Fig. 2. Effect of methanol (1.2–7.5 μl ml^{-1}) and decadienal in methanol on the development of embryos of *D. pulicaria* in vitro. Development success is defined by shedding of the first egg membrane.

producers. Compared with the marine *T. rotula, Fragilaria* sp. produces similar patterns of PUA in comparable quantities, if the smaller cell size of *Fragilaria* sp. is taken into account. While the larger *T. rotula* can produce approximately 4 fmol cell^{-1} (Pohnert et al. 2002), *Fragilaria* sp. used in this study releases 0.1 fmol cell^{-1} $\pm$ 0.01 (n = 3) of PUA upon wounding. Thus, one might expect similar effects on both copepods and *Daphnia. A. formosa* can be indirectly compared with the marine diatoms. In sea urchin egg-cleavage tests, the main PUA of this alga (12-ODTE) is 10 times less active than decadienal but acts presumably via the same inhibitory mechanism (Adolph et al. 2003). Because the production of 12-ODTE by *A. formosa* is 10-fold higher (49 fmol cell^{-1}) (Pohnert 2000) than the production of decadienal by *T. rotula,* the toxic potential of these species should again be comparable. 9-ODTE found in *G. parvulum* proved to be inactive in sea urchin egg cleavage assays, presumably due to intramolecular inactivation (Adolph et al. 2003). We found no evidence for any direct toxic effect of the PUA on g_j. *A. formosa* provides good growth and the two *Fragilaria* species are poor food, but the resulting growth rates do not differ significantly despite the presence or absence of aldehydes. Carotenuto and Lampert (2004) used the same argument to exclude the possibility that the aldehydes acted as feeding deterrents, as the ingestion rates did not differ either.

The differing response of *Daphnia* to diatom food in the long-term experiments may have been caused by differences in nutritional quality not detected in the short-term feeding measurements (Carotenuto and Lampert 2004). These differences can comprise a lack of polyunsaturated fatty acids (PUFAs) (Brett and Müller-Navarra 1997) or stoichiometric imbalance (DeMott and Tessier 2002). Evidence for effects of nutritional quality comes from the experiment with mixed diets. In every single case, g_j was higher when *Scenedesmus* replaced half of the diatoms. DeMott (1998) has demonstrated that different algae can complement each other with respect to nutritional quality. A combination of a diatom with *Scenedesmus* may, thus, enhance the overall nutritional balance of the diatoms and the growth rate of *D. pulicaria.*

There is no evidence for an effect of PUA on the survival of adults, the lifetime fecundity (R_0), and the instantaneous rate of population growth (r). The latter is a good measure of fitness (Stearns 1992), and besides age-specific survival and fecundity, it incorporates the viability of the eggs.

We observed reduced hatching of *Daphnia* eggs only for a single diet, pure *Fragilaria* sp., and only for late clutches (after the fourth brood). Although we can again not exclude a nutritional effect with increasing age of the female (all eggs hatched in the mixed diet), the fact that hatching is not affected by *F. capucina* provides evidence for the role of aldehydes produced by *Fragilaria* sp. On the contrary, no effect of the structurally related oxo-acids produced by *A. formosa* and *G. parvulum* was detected. This evidence is supported by the in vitro incubation of *Daphnia* eggs with different oxylipins. While 2,4-decadienal (produced by *Fragilaria* sp.) caused a concentration-dependent cessation of egg development, no such effect was found for 12-ODTE from *A. formosa.* The in vitro effect of 2,4-decadienal is similar as demonstrated for copepod egg hatching and cleavage of sea urchin eggs (Miralto et al. 1999). The lack of an effect with 12-ODTE may be explained by the much lower biological activity of this compound in the sea urchin test (Adolph et al. 2003). Although we detected one possible effect of aldehydes on *Daphnia* (reduced egg viability in late clutches), there is no evidence yet that the production of PUA has an ecological meaning for *Daphnia*–diatom interactions. The contribution of successive clutches to individual fitness is very different in copepods and *Daphnia* due to their different life histories. Copepods spawn in a relatively short time period, have longer larval development times, and there is no overlap in reproduction between females and their offspring. On the contrary, daphniids have a short juvenile phase and overlapping generations. Therefore, all clutches of copepods contribute equally to the next generation, but in *Daphnia,* the first clutches are more important than later clutches (Stearns 1992). Eggs of clutches 4 and later contribute very little to r, consequently, we found no difference in r for diets of *Fragilaria* sp., *F. capucina,* and *Scenedesmus,* despite egg mortality in late clutches with *Fragilaria* sp. diet. Inhibition of egg viability in late clutches cannot be a selective force for the evolution of a grazing defense against *Daphnia,* contrary to copepods. However, we cannot fully exclude the possibility that PUA-producing diatoms can reduce *Daphnia* fitness. Thus, e.g., females fed diatoms from birth on could produce nonviable eggs in earlier clutches or the survival of juveniles could be reduced by a maternal effect as recently demonstrated for copepods (Ianora et al. 2004).

Maintaining the enzymatic tools for the wound-activated production of oxylipins in diatoms may be costly. Thus, it remains an interesting research topic to identify the function of these compounds in providing an advantage to oxylipin-producing diatoms. Although freshwater copepods are not as important grazers as *Daphnia* and diatoms are not a domi-

nant component of their diet, they may eventually be affected like their marine relatives.

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Angewandte Chemie

Bioorganische Chemie

Neue Lipoxygenase-/Hydroperoxid-Lyase-Biosynthesewege im Moos *Physcomitrella patens***

*Thomas Wichard, Cornelia Göbel, Ivo Feussner und Georg Pohnert**

Lipoxygenasen (LOX) sind an der Biosynthese von zentralen Signal- und Verteidigungsmetaboliten in Säugetieren, höheren Pflanzen und Algen beteiligt.[1–4] Durch LOX katalysierte Biosynthesewege sind generell mit der Einführung von molekularem Sauerstoff in eine mehrfach ungesättigte Fettsäure verbunden. Die daraus resultierenden Hydroperoxide können zu kürzerkettigen sauerstoffhaltigen Produkten gespalten werden. Interessanterweise werden unterschiedliche Fettsäuren zur Produktion von Hydroperoxiden genutzt. Während Oxylipine in Pflanzen fast ausschließlich aus C_{18}-Fettsäuren generiert werden,[1] nutzen sowohl Säugetiere als auch Algen bevorzugt C_{20}-Fettsäuren.[3,5] Auch die weitere Umsetzung der intermediär auftretenden Hydroperoxide unterscheidet sich grundlegend: Aus Säugetieren sind bifunktionelle LOX bekannt, die auch die Hydroperoxidspaltung katalysieren, wogegen Pflanzen und Algen häufig Hydroperoxid-Lyasen (HPL) zur Generierung kürzerkettiger Oxylipine nutzen.[1–3] Wegen ihrer zentralen Stellung im Metabolismus sind LOX/HPL-Biosynthesewege in diesen Organismen gut untersucht. Dagegen ist nahezu nichts über verwandte Umsetzungen in Moosen bekannt. Da Moose phylogenetisch zwischen höheren Pflanzen und Algen eingeordnet werden, sind sie für die Untersuchung von LOX-Biosynthesewegen besonders interessant.

Von einigen Moosen ist bereits bekannt, dass sie unverzweigte C_8- und C_9-Alkohole sowie C_8- und C_9-Aldehyde über bisher unbekannte Biosynthesewege bilden.[6,7] Bei der Untersuchung von *Physcomitrella patens* fanden wir, dass auch dieses Laubmoos nach einer Gewebeverletzung flüchtige Oxylipine freisetzt. Darunter findet sich (*E*)-Non-2-enal (**4**), ein aus höheren Pflanzen bekannter, intensiv nach Gurken riechender Metabolit. Aber auch (*R*)-Oct-1-en-3-ol (**1**; 94% *ee*),[8] eine Hauptaromakomponente aus Pilzen, und (*E*)-Oct-2-en-1-ol (**2**) sowie (*E*)-Oct-2-enal (**3**) konnten detektiert werden (Abbildung 1). (*R*)-**1** wird in Pilzen aus Linolsäure

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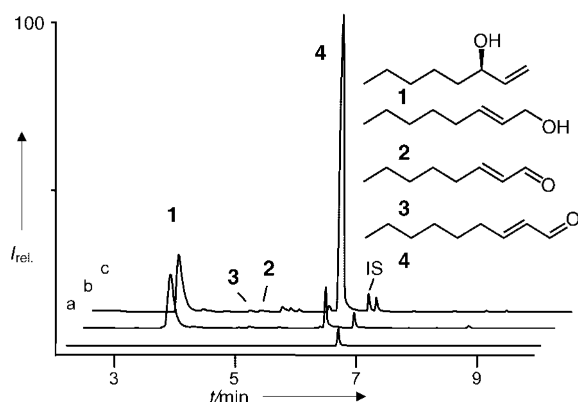


Abbildung 1. a) GC/MS des SPM-Extrakts (SPME = solid-phase microextraction, Festphasenmikroextraktion) von flüchtigen Verbindungen aus *P. patens*; b) wie a) aus dem verwundeten Moos; c) wie b) in Gegenwart von 200 µg Arachidonsäure pro ca. 100 mg *P. patens*. IS = Interner Standard; 2-Decanon.

generiert, die zunächst durch eine 10-LOX zum entsprechenden Hydroperoxid umgesetzt und dann durch eine Lyaseaktivität gespalten wird; dabei entsteht (*E*)-10-Oxodec-8-ensäure als zweites Bruchstück.[9–11] In höheren Pflanzen ist Linolsäure eine Vorstufe bei der Biosynthese von (*E*)-Non-2-enal (**4**) und (*Z*)-Non-3-enal (**8**). Hier sind eine 9-LOX und eine 9-HPL an der Produktion des C_9-Aldehyds **8** und der 9-Oxononansäure beteiligt.[2,12]

Wir fanden, dass eine Verwundung von *P. patens* in Gegenwart von Linol- oder α-Linolensäure nicht zu einer signifikant erhöhten Produktion von **1**–**4** oder verwandten höher ungesättigten Metaboliten führt. Auch 10-Oxodec-8-ensäure oder 9-Oxononansäure, Verbindungen, die in Pflanzen oder Pilzen durch vergleichbare Umsetzungen gebildet werden, konnten in den Moospräparationen nicht nachgewiesen werden. Diese Ergebnisse zeigen, dass Moose offensichtlich andere Vorstufen zur Bildung von **1**–**4** nutzen. Anders als höhere Pflanzen enthält *P. patens* auch Arachidonsäure (**5**),[13] die wegen der Positionen der Doppelbindungen ebenfalls eine potenzielle Vorstufe der kurzkettigen Oxylipine sein könnte. Tatsächlich wird extern zugegebene Arachidonsäure (**5**) mit großer Effizienz zu **1**–**4** umgesetzt. Das wurde besonders bei der Umsetzung von [D_8]Arachidonsäure durch *P. patens* deutlich: Nach Zugabe dieser markierten Fettsäure werden deuteriertes **1** (> 400 % Einbaurate), **2**, **3** und **4** (> 1100 % Einbaurate) gebildet (Abbildung 1). Dieser überraschende Befund – Arachidonsäure (**5**) wurde bisher noch nicht als Vorstufe von **1**–**4** identifiziert – motivierte uns zu einer genaueren Untersuchung der involvierten Biosynthesewege. Abfangexperimenten zufolge ist eine Arachidonat-12-LOX an den Umsetzungen beteiligt. Durch das Reduktionsmittel Dimethylsulfid werden intermediär auftretende Hydroperoxide zu den entsprechenden Alkoholen umgesetzt, die keine Substrate für HPL sind.[3] Im intakten Moos fanden wir nach Reduktion keine endogenen hydroxylierten Fettsäuren, wohingegen schon Sekunden nach einer Verwundung (*S*)-12-Hydroxyarachidonsäure ((*S*)-12-HETE, 83 % *ee*), (*S*)-15-HETE (56 % *ee*) und Spuren von nahezu racemischer 11-HETE nachgewiesen werden konnten. Das entsprechende 11-Hydroperoxid, 11-HPETE, könnte formal auch eine Zwischenstufe auf dem Weg zu den C_9-Verbindungen sein, da es aber nur in geringen Mengen als Racemat vorkommt, handelt es sich eher um ein Autoxidationsprodukt der Arachidonsäure, das nicht aus enzymatischen Reaktionen hervorgegangen ist. Anders als die Menge an 11-HPETE steigt die Menge an 12-Hydroperoxyeicosatetraensäure (12-HPETE, **6**) während der ersten 30 Sekunden nach Zugabe von Arachidonsäure (**5**) zu verwundetem Moos stark an. Innerhalb der nächsten 16 Minuten – der Zeit, die auch für die Bildung der flüchtigen Verbindungen **1**–**4** und der Oxosäuren benötigt wird – nimmt die Menge an **6** dann wieder ab (siehe Hintergrundinformationen). Diese Ergebnisse lassen darauf schließen, dass (*S*)-12-HPETE aus der LOX-vermittelten Arachidonsäureoxidation ein Intermediat in der Biosynthese von **1**–**4** ist.

Zur weitergehenden Aufklärung des Mechanismus der Fettsäuretransformationen in *P. patens* haben wir die Natur der polaren Bruchstücke, die zusätzlich zu **1**–**4** aus der Spaltung von **6** resultieren, aufgeklärt. Die Identifizierung der Metaboliten gelang durch einen Vergleich der HPLC-MS-Chromatogramme von *P.-patens*-Extrakten, die vor und nach der Verwundung angefertigt wurden. Eine Gewebeverletzung führt zur Neubildung von zwei dominanten, von Fettsäuren abgeleiteten Verbindungen. Das UV-Spektrum der weniger polaren Komponente (λ_{max} = 279 nm) lässt auf eine α,β,γ,δ-ungesättigte Carbonylverbindung schließen. Das Massenspektrum dieser Verbindung stimmt mit dem einer oxidierten C_{12}-Säure wie (5*Z*,8*Z*,10*E*)-12-Oxododeca-5,8,10-triensäure (12-ODTE, **7**) überein (Abbildung 2). Eine Coinjektion mit synthetischem **7**[14] führte zum endgültigen Strukturnachweis. Bei der anderen neu entstandenen Verbindung handelt es sich um 11-Oxoundeca-5,9-diensäure (11-OUDE, **10**).[15]

Die identifizierten Strukturen führen zu der in Schema 1 wiedergegebenen, postulierten Biosynthese für die Oxylipine: Demzufolge wird **5** zunächst, ohne vorhergehende Kettenverkürzung durch z. B. β-Oxidation, zum Hydroperoxid **6** umgewandelt, das dann zu den Oxosäuren und flüchtigen Verbindungen gespalten wird. Diese Biosynthesewege konnten durch Versuche mit [D_8]Arachidonsäure bestätigt werden. Nach Applikation der markierten Fettsäure wiesen die Massenspektren von **6**, **7** und **10** Verschiebungen von + 8, + 6 oder + 5 Masseneinheiten auf (Abbildung 2 und Hintergrundinformationen). Die übereinstimmend hohen Isotopeneinbauraten in **6** und in die kürzerkettigen Oxylipine zeigen, dass **5** die Vorstufe aller in Schema 1 gezeigten Oxylipine ist.

P. patens nutzt also bisher unbekannte Biosynthesewege zur Produktion bekannter LOX/HPL-Produkte. Die Biosynthese von **1**, **2** und **4** wird dabei durch eine 12-LOX initiiert, die **6** als Substrat für HPL oder andere Fettsäure spaltende Enzyme zur Verfügung stellt. Die weitere Umsetzung von **6** führt zur Bildung der C_8-Metaboliten **1** und **2** sowie zu **7** als zweitem Bruchstück (Schema 1). Interessanterweise wurde **7** bereits aus Arachidonsäure-stimulierten menschlichen Blutplättchen nachgewiesen.[16] Dort fungiert diese Verbindung als Antagonist zu menschlichen Neutrophilen und interferiert wahrscheinlich mit der Leukotrien-B4-Bindungsstelle.[17] **7** wurde auch als Verteidigungsmetabolit in Diatomeen identi-

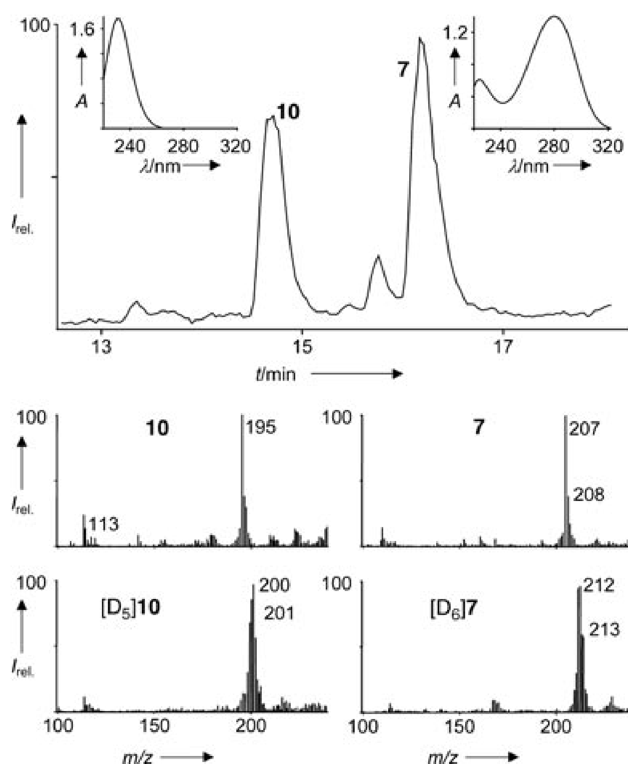
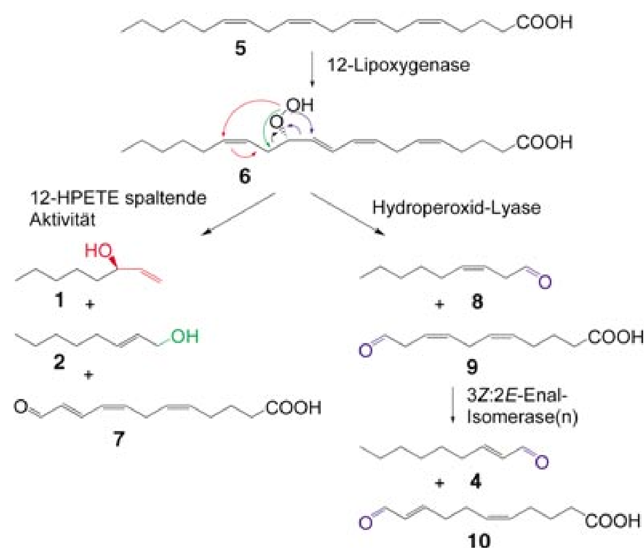


Abbildung 2. Oben: LC/MS eines Extrakts von *P. patens*, das in Gegenwart von 200 μg **5** pro ca. 100 mg Moos verwundet wurde. Die Einschübe geben die UV-Spektren von **7** und **10** wieder. Unten: Massenspektren bei negativer Ionisierung der unmarkierten (oben) und markierten (unten) Oxosäuren, die nach Umsetzung mit **5** bzw. $[D_8]$**5** entstehen.



Schema 1. Vorgeschlagene Arachidonat-12-LOX/HPL-Biosynthesewege in *P. patens*. In Gegenwart von $[D_8]$Arachidonsäure ($[D_8]$**5**) kann $[D_8]$HPETE ($[D_8]$**6**) abgefangen werden. In den weiteren Reaktionen werden $[D_2]$C8-Fragmente, $[D_6]$**7**, $[D_3]$**4** und $[D_5]$**10** gebildet. Alle acht Deuteriummarkierungen aus $[D_8]$**5** werden somit in den Produkten gefunden.

fiziert.[18,19] Von diesen Algen wird allerdings (1*E*,3*E*)-Octa-1,3-dien als zweites Bruchstück von 12-HPETE freigesetzt. Das Moos produziert **7** also über einen gänzlich anderen Biosyntheseweg als Diatomeen.[14] In *P. patens* kann **6** auch zu (*Z*)-Non-3-enal (**8**) und (5*Z*,8*Z*)-11-Oxoundeca-5,8-diensäure (**9**) umgesetzt werden. Diese Transformation wird wahrscheinlich durch eine HPL katalysiert. Ähnlich wie bei gut untersuchten Umsetzungen in höheren Pflanzen könnten diese Intermediate dann schnell durch eine 3*Z*:2*E*-Enal-Isomerase zu **4** und **10** umgewandelt werden (Schema 1).[12,20]

Unsere Ergebnisse zeigen, dass das Moos *P. patens* neue LOX-Biosynthesewege für die Produktion von Oxylipinen mit vielfältigen Strukturen nutzt. Es setzt Metaboliten frei, die als typisch für Säugetiere, Pflanzen, Pilze oder Algen angesehen wurden. In bisher nicht bekannter Weise werden biosynthetische Motive all dieser Organismen dazu genutzt, Arachidonsäure zu transformieren. Es ist bekannt, dass Moose ausgesprochen resistent gegen Herbivore und Pathogene sind. In höheren Pflanzen werden derartige Resistenzen oft durch Lipoxygenasen vermittelt.[21] Es wird in Zukunft von besonderem Interesse sein, herauszufinden, ob und wie die hier neu identifizierten Biosynthesewege zur Produktion von Signal- oder Verteidigungsmetaboliten genutzt werden und so zur Resistenz der Moose beitragen können.

Experimentelles

Allgemeine Methoden: *P.-patens*-Kulturen wurden von Prof. Dr. Ralf Reski, Freiburg, zur Verfügung gestellt und mit bereits beschriebenen Methoden kultiviert.[22] Zur Vorbereitung der Proben für die MS-Analyse wurden 5 mL einer dichten Kultur zum Sedimentieren stehen gelassen und das überstehende Medium mit einer Pasteurpipette entfernt. Das so erhaltene Pellet von 100 bis 200 mg Feuchtgewicht wurde in 1 mL frischem Medium resuspendiert und entweder direkt oder nach Behandlung mit 20 μL einer Lösung der jeweiligen Fettsäuren ($10\,mg\,mL^{-1}$ Ethanol) weiterverwendet. Die Suspensionen wurden 2 min im Eisbad mit einem Ultraschall-Desintegrator behandelt. Für die SPME-Analyse wurden die Probengefäße mit einem Teflonseptum versiegelt und 15 min mit einer Polydimethyldiloxanfaser (Supelco) extrahiert. Die GC/MS-Analyse erfolgte nach einer bekannten Methode.[14] Käufliche Standards wurden zur Identifizierung von **1–4** herangezogen. Teil der Probenvorbereitung für die LC/MS-Analyse war eine Ultraschallbehandlung wie oben beschrieben. 30 s oder 16 min nach Zellverletzung wurde 1 mL MeOH zugegeben, zentrifugiert (5 min, 12000 rpm) und der Überstand direkt zur Analyse an einer Agilent HP1100/Finnigan LCQ verwendet.

Die intermediären Hydroperoxide in den mit Arachidonsäure behandelten Suspensionen wurden durch Zugabe einer 10-proz. Dimethylsulfid-Lösung in MeOH reduziert. Der *ee*-Wert der resultierenden Alkohole wurde nach einer bekannten Methode bestimmt.[23] Mittelwerte aus Messungen nach 30 s, 2 min, 8 min, 15 min und 30 min wurden bestimmt.

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Stichwörter: Biosynthese · Fettsäuren · Massenspektrometrie · Oxylipine · UV/Vis-Spektroskopie

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Unprecedented Lipoxygenase/Hydroperoxide Lyase Pathways in the Moss *Physcomitrella patens***

*Thomas Wichard, Cornelia Göbel, Ivo Feussner, and Georg Pohnert**

Lipoxygenase (LOX) pathways are involved in the production of important signal and defensive metabolites in mammals, higher plants, and algae.[1–4] In these pathways molecular oxygen is introduced into a polyunsaturated fatty acid to form an intermediate hydroperoxide, which may then be cleaved to give shorter chain-length oxygenated products. Interestingly, different principles of transformations have been identified. While plants use almost exclusively C_{18} fatty acids for the production of oxylipins,[1] algae and animals rely predominantly on the transformation of C_{20} fatty acids.[3,5] In animals cleavage of the intermediate hydroperoxy fatty acids is achieved by a dual function of LOXes, while plants and algae rely often on hydroperoxide lyases (HPLs) to produce shorter chain oxylipins.[1–3] Due to their central importance, LOX/HPL pathways have been well investigated in these organisms, but nearly nothing is known about related transformations in mosses. Since mosses are located phylogenetically between higher plants and algae, the biosynthetic pathways are of special interest.

Certain mosses are known to release unbranched unsaturated C_8 and C_9 alcohols and aldehydes, but until now the pathways to and function of these metabolites have not been addressed.[6,7] We report here that the moss *Physcomitrella patens* releases volatile oxylipins upon tissue damage. These include (*E*)-non-2-enal (**4**) known from higher plants, which has a cucumberlike odor, (*R*)-1-octen-3-ol (**1**) (94% *ee*),[8] a major aroma compound from mushrooms, (*E*)-oct-2-en-1-ol (**2**), and (*E*)-oct-2-enal (**3**; Figure 1). In mushrooms (*R*)-**1** is derived from linoleic acid, which is transformed by a 10-LOX and a lyase activity to give (*E*)-10-oxodec-8-enoic acid as a second fragment.[9–11] In higher plants linoleic acid is also a precursor for the generation of (*E*)-non-2-enal (**4**) and (*Z*)-non-3-enal (**8**). There, a 9-LOX and a 9-HPL produce the C_9 aldehyde **8** and 9-oxononanoic acid.[2,12]

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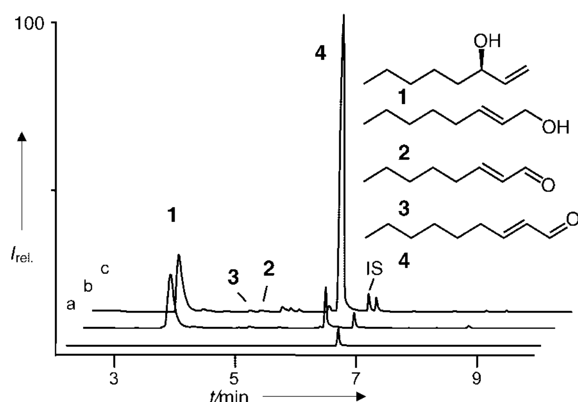

Figure 1. a) GC/MS of the SPM-extract of volatiles from *P. patens*; b) same as a) from the wounded moss; c) same as b) in the presence of 200 μg arachidonic acid (**5**)/~100 mg *P. patens*. IS = internal standard 2-decanone.

In our investigations on the biosynthesis of the volatile oxylipins from *P. patens* we found that external application of linoleic or α-linolenic acid did not result in significantly increased production of **1**–**4** or related higher unsaturated metabolites. Moreover, neither 10-oxodec-8-enoic acid nor 9-oxononanoic acid, metabolites formed from analogous pathways in plants and mushrooms, were detected. These results imply that mosses rely on other precursors for the generation of **1**–**4**. In contrast to higher plants, *P. patens* is also rich in arachidonic acid (**5**),[13] which could, judging from the position of the double bonds, also be a possible precursor for these volatile oxylipins. Indeed, externally applied arachidonic acid (**5**) was transformed with high efficiency into **1**–**4**. This could be shown impressively by administration of [D$_8$]arachidonic acid to *P. patens* cell preparations, which resulted in deuterated **1** (>400% labeled), **2**, **3**, and **4** (>1100% labeled) (Figure 1). This surprising fact—arachidonic acid (**5**) has not been identified as a precursor for **1**–**4** in any other organisms—prompted us to investigate the pathways in more detail. Trapping experiments with dimethyl sulfide revealed that an arachidonate 12-LOX is involved in the transformations. Using this reagent, intermediate hydroperoxy fatty acids are transformed into the corresponding alcohols, which are not further metabolized by HPL.[3] In intact moss we found no endogenous hydroxylated fatty acids after reduction; however, after only a few seconds after tissue damage, (*S*)-12-hydroxyarachidonic acid ((*S*)-12-HETE, 83% *ee*), (*S*)-15-HETE (56% *ee*), and minor amounts of nearly racemic 11-HETE were detected. The related 11-hydroperoxide, 11-HPETE, could also be a precursor for the C$_9$ volatiles, but since it is found only in minor amounts as a racemate, it is most likely an autoxidation product of arachidonic acid and not involved in enzymatic pathways. In the presence of **5** the amount of 12-hydroperoxyeicosatetraenoic acid (12-HPETE, **6**) increased drastically within 30 seconds after wounding and decreased over the next 16 minutes—the time required for the formation of **1**–**4** and oxo-acids (see the Supporting Information). Accordingly, (*S*)-12-HPETE, which arises from the LOX-mediated oxidation of arachidonic acid (**5**), is an intermediate in the biosynthesis of **1**–**4**.

To further investigate the mechanism of fatty acid transformation in *P. patens* we identified the fragments generated from **6** in addition to **1**–**4**. The HPLC-MS chromatograms obtained from *P. patens* preparations before and after tissue damage were used to identify two dominant metabolites derived from fatty acids. The UV spectrum (λ_{max} = 279 nm) of the less polar metabolite is characteristic for an α,β,γ,δ-unsaturated carbonyl compound, and the mass spectrum suggested an oxidized C$_{12}$ carboxylic acid (Figure 2). These

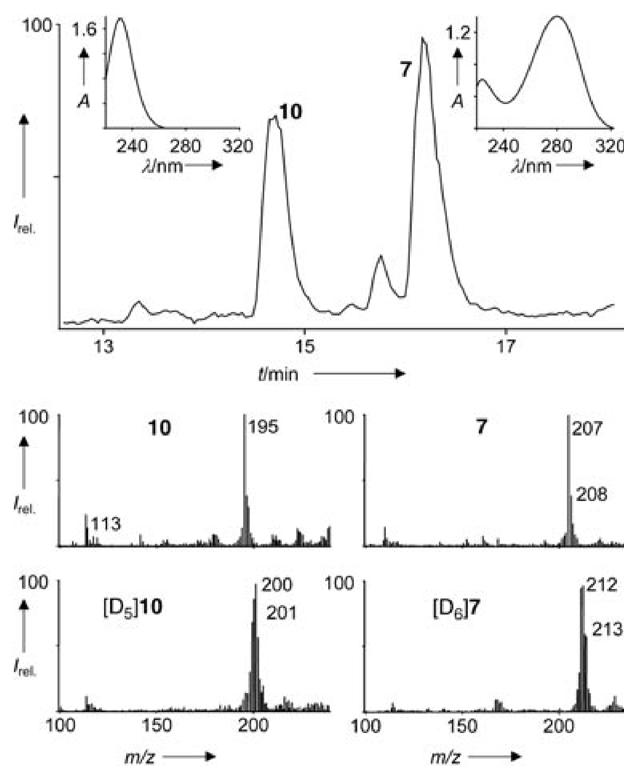

Figure 2. Top: LC/MS of wounded *P. patens* in the presence of 200 μg arachidonic acid (**5**)/~100 mg *P. patens*. The inserts show the UV-spectra of the metabolites **7** and **10**, respectively. Bottom: Negative ionization mass spectra for the unlabeled (above) and labeled (below) oxo-acids after transformation of **5** or [D$_8$]-**5**.

data matched those of (5*Z*,8*Z*,10*E*)-12-oxododeca-5,8,10-trienoic acid (12-ODTE, **7**) and co-injection with synthetic **7**[14] finally proved the structure. The other metabolites could be identified as 11-oxoundeca-5,9-dienoic acid (11-OUDE, **10**).[15]

These assigned structures are in accordance with biosynthetic considerations on the generation of the oxylipins shown in Scheme 1. The identified structures suggest that **5** is transformed without prior degradation by, for example, β-oxidation, into the hydroperoxide **6**, which then undergoes cleavage to give the oxo-acids and volatiles. This biosynthetic path was further supported by incubations of damaged moss with [D$_8$]arachidonic acid. Mass spectra of **6**, **7** and **10** showed mass shifts of +8, +6 and +5, respectively, when the labeled acid was present (see Figure 2 and the Supporting

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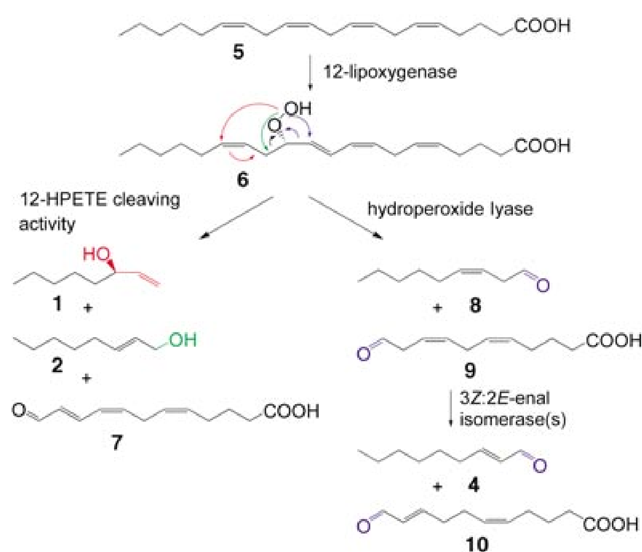

Scheme 1. Proposed arachidonate 12-LOX/HPL pathways in *P. patens*. After administration of [D$_8$]-arachidonic acid (**5**) [D$_8$]-HPETE (**6**) can be trapped as intermediate. [D$_2$]-C8-fragments and [D$_6$]-**7** as well as [D$_3$]-**4** and [D$_5$]-**10** are formed in the subsequent reactions. All 8 deuterium labels of **5** are thus found in the respective intermediates and break down products.

Information). The corresponding high degree of labeling of **6** and the shorter chain oxylipins indicates that **5** is the precursor of all the oxylipins shown in Scheme 1.

These results show that *P. patens* employs hitherto unknown pathways for the production of known LOX/HPL products. The biosynthesis of **1**, **2**, and **4** is initiated by a 12-LOX, which provides **6** as a substrate for HPL or other fatty acid cleaving enzymes. Further transformation of **6** results in the C_8 metabolites **1** and **2** together with **7** as the second fragment (Scheme 1). Interestingly, **7** has been detected previously in arachidonic acid stimulated human platelets.[16] It acts as an agonist towards human neutrophils, presumably interacting with the leukotriene B4 binding site.[17] Compound **7** is also known as deleterious metabolite from diatoms,[18,19] but there (1*E*,3*E*)-octa-1,3-diene results as the second fragment from a 12HPETE.[14] In *P. patens* **6** can also be transformed into (*Z*)-non-3-enal (**8**) and (5*Z*,8*Z*)-11-oxoundeca-5,8-dienoic acid (**9**), presumably by an HPL. In analogy to well-known transformations in higher plants, these intermediates may be transformed quickly by a 3*Z*:2*E*-enal isomerase to give **4** and **10** (Scheme 1).[12,20]

Our findings show that hitherto unknown LOX pathways are involved in the biosynthesis of a multitude of oxylipins in *P. patens*. The moss produces metabolites typical for animals, plants, algae, and mushrooms by new transformations of arachidonic acid, combining in a unique way metabolic themes from all these organisms. Mosses are known to be highly resistant to herbivores and pathogens, and in higher plants this type of resistance is often mediated by lipoxygenases.[21] We are particularly interested in determining if and how the newly identified biosynthetic pathways contribute to the production of putative signal or defensive metabolites involved in this remarkable resistance.

Experimental Section

General: Cultures of *P. patens* were obtained from Prof. Dr. Ralf Reski, Freiburg, and were cultivated as described.[22] Preparation of samples for MS analysis: A 5-mL aliquot of a dense culture was left to settle, and the medium was removed by pipetting. The resulting pellet with a wet weight of 100–200 mg was resuspended in 1 mL of fresh medium and used directly or treated with 20 µL of 10 $mg\,mL^{-1}$ solutions of fatty acids in EtOH. The suspension was sonicated and cooled in an ice bath for 2 min. For SPME analysis the vial was sealed with a Teflon septum, and a polydimethyldiloxane-coated fiber (Supelco, Taufkirchen, Germany) was inserted for 15 min in the gas phase. Analysis by GC/MS was performed as described.[14] Commercially available reference compounds were used for the identification of **1**–**4**. For LC/MS analysis, the moss preparation was treated 30 s or 16 min after sonication with the same volume of MeOH. The sample was centrifuged for 5 min (12000 rpm), and the supernatant was used directly for analysis on an Agilent HP1100/Finnigan LCQ.

The intermediate hydroperoxy fatty acids were trapped by addition of a 10% solution of dimethyl sulfide in methanol to preparations previously treated with arachidonic acid (**5**). The *ee* value was determined by a known method.[23] For calculation, means of measurements after 30 s, 2 min, 8 min, 15 min, and 30 min were used.

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A Multifunctional Lipoxygenase with Fatty Acid Hydroperoxide Cleaving Activity from the Moss *Physcomitrella patens**

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A complex mixture of fatty acid-derived aldehydes, ketones, and alcohols is released upon wounding of the moss *Physcomitrella patens*. To investigate the formation of these oxylipins at the molecular level we isolated a lipoxygenase from *P. patens,* which was identified in an EST library by sequence homology to lipoxygenases from plants. Sequence analysis of the cDNA showed that it exhibits a domain structure similar to that of type2 lipoxygenases from plants, harboring an N-terminal import signal for chloroplasts. The recombinant protein was identified as arachidonate 12-lipoxygenase and linoleate 13-lipoxygenase with a preference for arachidonic acid and eicosapentaenoic acid. In contrast to any other lipoxygenase cloned so far, this enzyme exhibited in addition an unusual high hydroperoxidase and also a fatty acid chain-cleaving lyase activity. Because of these unique features the pronounced formation of (2*Z*)-octen-1-ol, 1-octen-3-ol, the dienal (5*Z*,8*Z*,10*E*)-12-oxo-dodecatrienoic acid and 12-keto eicosatetraenoic acid was observed when arachidonic acid was administered as substrate. 12-Hydroperoxy eicosatetraenoic acid was found to be only a minor product. Moreover, the *P. patens* LOX has a relaxed substrate tolerance accepting C_{18}-C_{22} fatty acids giving rise to even more LOX-derived products. In contrast to other lipoxygenases a highly diverse product spectrum is formed by a single enzyme accounting for most of the observed oxylipins produced by the moss. This single enzyme might, in a fast and effective way, be involved in the formation of signal and/or defense molecules thus contributing to the broad resistance of mosses against pathogens.

Lipoxygenase (LOX)[1] pathways are involved in the production of important hormones and defensive metabolites in plants, algae, and animals (1–3). Metabolites originating from these pathways are collectively named oxylipins. In general LOX constitute a large gene family of non-heme iron-containing dioxygenases, which are ubiquitous in higher plants and animals (3). This group of enzymes catalyzes the regio- and stereospecific insertion of dioxygen into polyunsaturated fatty acids that harbor a (1*Z*,4*Z*)-pentadiene system resulting in a (2*E*,4*Z*)-hydroperoxy diene structural element (1). These hydroperoxides may be subsequently cleaved to shorter chain length oxygenated products by LOX themselves or by hydroperoxide lyases (HPL) (4, 5). The cleavage products include volatile unsaturated aldehydes and alcohols and the corresponding unsaturated oxo fatty acids (1, 5). Because of their central importance in signal and defense processes, the biosynthesis of oxylipins is well investigated in higher plants and animals as well as in algae. Interestingly, different principles of transformations have been found in these genera. While higher plants use exclusively polyunsaturated C_{18} fatty acids for the production of oxylipins, animals and algae rely predominantly on the transformation of polyunsaturated C_{20} fatty acids (3, 6), which are not ubiquitously found in the plant kingdom (7). Moreover, the formation of volatile short chain aldehydes relies on the combined action of LOX and HPL species in higher plants, whereas animals and algae seem to be more flexible, because they may use either the LOX/HPL system or specific LOX forms alone (6, 8, 9). Numerous cDNAs corresponding to LOX have been isolated and characterized from higher plants and animals, but molecular data and mechanistic insight into LOX from other organisms are still scarce (10–12).

It is known that mosses release volatile C_8 and C_9 alcohols and aldehydes with hitherto unknown biosynthesis and function (13). Mosses are of special interest with respect to their LOX-derived chemistry because they diverged more than 400 million years ago from flowering plants, and it is discussed to which extent their metabolism has evolved independently (14). Recently, we described an investigation of LOX-derived products in the moss *Physcomitrella patens*, which is a widely used model organism in this context (15). We found that this moss releases the volatile oxylipins 1-octen-3-ol, 2-octen-1-ol, and (2*E*)-nonen-2-al upon tissue disruption (16). 1-Octen-3-ol is a well known aroma compound from mushrooms and the C-9 aldehyde accounts with cucumber-like odor to the volatile

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ583499.

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[1] The abbreviations used are: LOX, lipoxygenase; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; HPLC, high performance liquid chromatography; RP, reversed phase; HPL, hydroperoxide lyase; H(P)ODE, hydro(pero)xy octadecadienoic acid; H(P)OTE, hydro(pero)xy octadecatrienoic acid; H(P)ETE, hydro(pero)xy eicosatetraenoic acid; H(P)EPE, hydro(pero)xy eicosapentaenoic acid; H(P)DPE, hydro(pero)xy docosapentaenoic acid; H(P)DHE, hydro-(pero)xy docosahexaenoic acid; KETE, keto eicosatetraenoic acid; ODTE, oxo dodecatrienoic acid; PpLOX, *P. patens* lipoxygenase; BSTFA, *N,O-bis*-(trimethylsilyl)-trifluoroacetamide; MSTFA, *N*-methyl-*N*-trifluoroacetamide; TOF, time of flight; ESI, electrospray ionization.

This paper is available on line at http://www.jbc.org

blend of certain plants. It is generally assumed that these metabolites derive from the LOX/HPL-mediated transformation of C_{18}-polyunsaturated fatty acids. In mushrooms, linoleic acid serves as precursor for the biosynthesis of 1-octen-3-ol while α-linolenic acid provides for the higher unsaturated alcohols (2, 5)-octadien-1-ol and (1, 5)-octadien-3-ol (17). In these cases the 10-hydroperoxy fatty acids are intermediates and 10-oxo-decenoic acid results as second fragment (18–20). Higher plants exploit a 9-LOX/HPL mechanism for the formation of 3*Z*-nonenal, which is subsequently transformed to the 2*E*-isomer (5). However, it should be stressed that the efficiency of HPL is low and represents the rate-limiting step in this process (5). Data on the involvement of conjugated unsaturated aldehydes in plant defense are scarce and they are still discussed controversially.

Some protein preparations containing LOX activity from mammals and plants have been described that not only introduce dioxygen into the fatty acid, but moreover exhibit hydroperoxide cleaving activity releasing unsaturated aldehydes. An HPL-like activity has been attributed to a LOX purified from mammalian leukocytes, which results in the formation of (5*Z*,8*Z*,10*E*)-12-oxo-dodeca-5,8,10-trienoic acid, but this activity has not been assigned to a single enzyme as yet at the molecular level (8).

Interestingly, we found that the moss does not follow any of the known pathways for the generation of the volatile oxylipins, but rather exploits C_{20}-polyunsaturated fatty acids as precursors for C_8- and C_9-alkenals and alkenols. As second fragments (5*Z*,8*Z*,10*E*)-12-oxo-dodeca-5,8,10-trienoic acid and (5*Z*,8*Z*)-11-oxo-undeca-5,8-dienoic acid are generated (16). Thus, the moss produces mixtures of typical plant, animal, and mushroom metabolites by hitherto unknown pathways from arachidonic acid, but meets metabolic themes from algae as well.

The unusual biosynthesis of *P. patens* oxylipins prompted us to question the metabolic pathways behind the generation of the metabolites. Judging form the product spectrum, LOX/HPL pathways as well as the action of a LOX alone, had to be taken into account.

In this study we report the identification, cloning, heterologous expression, and functional characterization of the first LOX from mosses. Phylogenetic analysis showed that it groups with plant LOX whereas the enzymatic activity and substrate preference resembles more that of animal LOX. Most interestingly, this LOX from *P. patens* exhibits an unusual high hydroperoxidase and lyase activity accounting for several of the moss oxylipins and might thus be regarded as the first of a series of related key enzymes involved in the production of reactive aldehydes and alcohols in mosses.

EXPERIMENTAL PROCEDURES

Materials—The chemicals used were from the following sources: fatty acids and standards of chiral and racemic hydroxy fatty acids as well as keto fatty acids were from Cayman Chemical (Ann Arbor, MI), eicosapentaenoic acid and docosahexaenoic acid were from Sigma, 2-octen-1-ol was obtained from Lancaster (Lancashire, England), methanol, hexane, 2-propyl alcohol (all HPLC grade) were from Baker; and restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Isolation of PpLOX1—An EST library from *P. patens* was provided by BASF Plant Science (21) and a partial EST clone with a putative LOX sequence was identified based on sequence homology to higher plant LOX. To obtain the 5′-terminus of the cDNA, a 5′-end RACE was performed using the gene-specific 3′ primer A (GAG CCC CTG TCT TCT CGG TAT TG) and the vector-specific 5′ primer T3. The PCR reaction was carried out with the *Tfl* polymerase (Biozym, Hessisch Oldendorf, Germany) under standard conditions. The obtained RACE fragment was sequenced. To clone the complete open reading frame, the RACE fragment was amplified from the cDNA-library using the gene-specific 5′ primer B (GGT ACC ATG ATG CTC AAC CGG TTG AC, GAT GCT GTT GGG CAC, HindIII site underlined). The obtained fragments were each cloned into pGEM-T (Promega, Mannheim, Germany). For expression in *Escherichia coli*, the complete open reading frame was assembled in pQE30 (Qiagen, Hilden, Germany) from both fragments using a common SalI site. The 3′-terminus was cloned first into pQE30 as a SalI/HindIII fragment and joined with the 5′-terminus by cloning the latter as a KpnI/SalI fragment into this construct to yield pQE-PpLOX1 (the nucleotide sequence has been submitted to the GenBank™ with accession number AJ583499), and the plasmid was transformed in *E. coli* strain SG13009. Control experiments were performed with the same strain transformed with pQE30 alone.

Sequence Analysis—Phylogenetic tree analysis was performed on amino acid sequences of selected LOX and PpLOX1 using phylip 3.5 according to Ref. 1. Prediction of the cleavage site of the chloroplastidic transit peptide was performed with ChloroP.

Protein Expression and LOX Activity Assay—Expression of pQE-PpLOX1 was performed as described for plant LOX (22). For product analysis 0.8 ml cell lysate was incubated with the respective fatty acid in 1.2 ml of 100 mM Tris buffer, pH 8.1, for 30 min at room temperature. Reactions were stopped by adding 9 mM $SnCl_2$ or 10% DMS in methanol (by volume) and incubation for 10 min to reduce hydroperoxy fatty acids to the corresponding hydroxides. The samples were acidified to pH 3, and the lipids were extracted according to Ref. 23. The obtained lipids were dissolved in 0.1 ml of methanol, and aliquots were submitted to HPLC analysis.

Analysis of Hydroperoxy Fatty Acids—HPLC analysis was carried out after reduction with $SnCl_2$ on an Agilent (Waldbronn, Germany) 1100 HPLC system equipped with a diode array detector. Oxygenated fatty acids were separated from unconverted fatty acids by RP-HPLC using a Nucleosil C18 column (250 × 4 mm, 5-μm particle size; Macherey-Nagel, Düren, Germany) with a solvent system of methanol/water/acetic acid (85:15:0.1, by volume) at a flow rate of 1 ml min^{-1}. Absorption at 234 nm (conjugated diene system of the hydroxy fatty acids) and 210 nm (polyenoic fatty acids) were recorded simultaneously. Straight phase HPLC was used to separate the hydroxy fatty acid isomers and carried out on a Zorbax RX-SIL column (150 × 2.1 mm, 5-μm particle size; Agilent) with a solvent system of *n*-hexane/2-propyl alcohol/acetic acid (100:1:0.1, by volume) at a flow rate of 0.2 ml min^{-1}. The enantiomeric composition of the hydroxy fatty acids was analyzed by chiral phase HPLC on a Chiralcel OD-H column (150 × 2.1 mm, 5-μm particle size; Daicel Chemical Industries, Illkirch, France) with a solvent system of *n*-hexane/2-propyl alcohol/acetic acid (100:2:0.1, by volume) at a flow rate of 0.1 ml min^{-1}.

Hydroxy fatty acids, for which standards are not commercially available, were verified by gas chromatography/mass spectrometry. Accordingly, the hydroxy fatty acids were methylated in methanol with carbodiimide solution (24). After extraction of the hydroxy fatty acid methyl esters with hexane, the samples were evaporated to dryness, dissolved in 3 μl of acetonitrile, and trimethylsilylated with 1 μl of *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) according to the manufacturer's instructions (Pierce). The analysis was carried out using an Agilent 5973 Network mass selective detector connected to an Agilent 6890 gas chromatograph equipped with a capillary DB-23 column (30 m × 0.25 mm; 0.25-μm coating thickness; J&W Scientific, Agilent). Helium was used as a carrier gas (1 ml min^{-1}). The temperature was ramped from 60 to 110 °C at 25 °C min^{-1} (held for 1 min), then 110–270 °C at 10 °C min^{-1} (held for 10 min).

Analysis of Lyase Products and Keto Acids—Product analysis of oxo and keto acids was performed by reversed-phase HPLC/MS (Hewlett-Packard Series II 1100 system equipped with a diode array UV detector coupled to a Thermo Finnigan LCQ (San Jose, CA) electrospray ionization (ESI) ion trap mass spectrometer). A nucleosil C18 column (ODS-3, 125 mm × 2 mm GromSil) was used for HPLC separation of the LOX/HPL-products with a binary gradient system of water/acetic acid (100:0.5, by volume) and acetonitrile/acetic acid (100:0.5, by volume) at a flow rate of 0.2 ml min^{-1}. Absorption at 237 nm (conjugated diene system of the hydroperoxy fatty acids) and at 279 nm (conjugated ketodienoic acids and $\alpha,\beta,\gamma,\delta$-unsaturated ω-oxo acids) were recorded simultaneously.

Volatile products for GC/MS analysis were extracted either from 1 ml of lysate prepared as described above by solid phase micro-extraction (SPME) or with 0.5 ml of $CHCl_3$. For SPME a polydimethylsiloxane-coated fiber (Supelco, Bellefonte, CA) was used as described (25). Solvent extracts were dried over Na_2SO_4, concentrated, treated with 10 μl MSTFA, and incubated at 50 °C for 30 min. Analysis was performed on

a Thermo Finnigan Trace GC/MS equipped with an Alltech (Deerfield, IL) EC5-MS column (15 m × 0.25 mm; 0.25-μm coating thickness). The temperature was ramped from 60 °C (2 min) to 100 °C at 5 °C min^{-1} and then to 300 °C at 25 °C min^{-1} (held for 2 min). For high resolution GC/MS, a three sector double focusing spectrometer MassSpec2 and a GC-TOF (Micromass, Manchester, England) were used.

Time Course—The enzyme assay was performed in 5-ml glass vials and started by addition of 0.5 mM arachidonic acid (10 mg ml^{-1} in EtOH) to 2 ml lysate (pH 8.1). After defined time spans, the reaction was stopped by addition of 200-μl aliquots to equal volumes of methanol and freezing in liquid nitrogen. After thawing and centrifugation, the supernatant was analyzed by RP-HPLC-ESI/MS. For quantification, the area (UV) of the detected substances was determined and normalized with the extinction coefficients (12-ODTE, ϵ = 38,700 liters mol^{-1} cm^{-1} ($\alpha,\beta,\gamma,\delta$-unsaturated aldehyde); 12-KETE, ϵ = 30,000 liters mol^{-1} cm^{-1}; 12-HPETE, ϵ = 27,000 liters mol^{-1} cm^{-1}). The analysis of remaining arachidonic acid was performed by GC/MS after addition of [$^2H_{27}$]myristic acid (20 μg ml^{-1} in EtOH) as internal standard and esterification with acetyl chloride/MeOH (26). At 30 s, 2, 8, and 30 min after the start of the experiments, additional samples were taken to determine the enantiomeric composition of 12-HPETE as described above.

RESULTS

Cloning and Expression of a LOX from P. patens—To gain insight into the nature of enzymes involved in unsaturated aldehyde and alcohol production in mosses, a sequenced EST library from *P. patens* (21) was analyzed. Data base searches and alignments of the EST clones revealed a cDNA of 1336 bp with significant similarity to plant LOX. The corresponding amino acid sequence of this cDNA exhibited highest identity to LOX1 from *Pisum sativum* (GenBank™ accession no. X07807, 44%). To isolate the full-length cDNA clone, RACE with specific primers was used for amplification of the missing 5′-end. The RACE fragment was fused to the 3′-terminus of the cDNA. The obtained complete open reading frame was named PpLOX1 (GenBank™ accession no. AJ583499) and had a length of 2814 bp encoding a protein of 937 amino acids with a molecular size of 105 kDa. The entire sequence showed highest identities to AtLOX3 from *Arabidopsis thaliana* (GenBank™ accession no. CAB56692, 43%) and POTLX-2 from *Solanum tuberosum* (GenBank™ accession no. AAB67860, 43%). The protein sequence alignment with these two plant LOX is presented in Fig. 1. A low sequence similarity was found within the first 90 amino acids that are enriched in serine and threonine in the case of PpLOX1 and AtLOX3 representing the putative transit peptide region for chloroplast import, which is typical for type2-LOX (1). Additional sequence analysis shows that the central histidine-rich region around His-597 and His-602 and the distal histidine (His-789), described for all plant LOX cDNAs sequenced so far, are conserved in the PpLOX1 amino acid sequence as well. As described for other enzymes from higher plants, an asparagine (Asn-793), an isoleucine (Ile-937) as well as the three histidine residues are involved in iron-ligand binding (27). Further, sequence-based analysis of the secondary structure revealed the typical organization of LOX with the catalytic domain at the C terminus and an N-terminal C2 domain (Fig. 2*A*). The C2 domain of PpLOX1 as well as of plant LOX is longer than the corresponding C2 domain of mammalian LOX.

A phylogenetic tree analysis of PpLOX1 and LOX from plants, mammals, algae, and corals showed that PpLOX1 grouped together with the type2-13-LOX from *A. thaliana*. The next closely related enzymes are type1-9-LOX from plants whereas mammalian LOX that exhibit the same substrate- and regiospecificity as PpLOX1 (see below) are clearly separated (Fig. 2*B*).

Lipoxygenase Activity—For further characterization PpLOX1 was expressed in *E. coli*. Functional analysis revealed that the enzyme had a broad substrate tolerance and metabolized administered free fatty acids with chain lengths ranging from 18 to 22 carbons. In control experiments with *E. coli* containing only the vector pQE30, no significant LOX activity was observed. The pH optimum of the PpLOX1 determined by summing up the integrals of all isomeric hydroperoxy fatty acids formed out of linoleic acid was between 8.0 and 8.2. This was also the pH range in which the highest regiospecificity was observed (83% 13-HPODE at pH 8.2; 65% 13-HPODE at pH 5.6).

13-HPODE, 13-α-HPOTE, 10-γ-HPOTE, 12-HPETE, 12-HPEPE, and 14-HPDHE were the preferred products after addition of linoleic acid, α-linolenic acid, γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, and (to challenge substrate tolerance) docosahexaenoic acid, respectively (Table I). The *S*-enantiomers dominated in all of these major products. The C_{20} fatty acids arachidonic acid and eicosapentaenoic acid were the preferred substrates for PpLOX1. Compared with these C_{20} fatty acids C_{22}-homologues were transformed with 68% efficiency, and the efficiency for C_{18} fatty acids was between 48% for γ-linolenic acid and 29% for α-linolenic acid, respectively. Transformation of eicosapentaenoic acid and arachidonic acid resulted in hydroperoxy fatty acid formation with highest regio- and stereoselectivity (Table I). Taken together these properties specify PpLOX1 as arachidonate 12*S*-LOX. In the case of γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid dioxygen was introduced preferably in position ω-9. Linoleic acid and α-linolenic acid, where this position is not able to be attacked by a LOX, were preferentially oxidized in position ω-6. PpLOX1 thus exhibits linoleate 13*S*-LOX activity as well. Generally, dioxygen introduction was favored at the [+2] position from a doubly allylic methylene with respect to the hydrogen abstraction site. Fatty acid methyl esters or fatty acids in glycerolipids were not substrates of PpLOX1.

Hydroperoxidase and Lyase Activity—In the presence of elevated substrate concentrations (≥0.4 mM), PpLOX1 exhibited pronounced hydroperoxidase and lyase activity. Shortly after the addition of arachidonic acid, 12-HPETE (Fig. 3, *peak 1*) dominates, but a rapid onset of the production of additional metabolites with UV maxima at 279 nm was observed (Fig. 3). The pseudo-molecular ions (ESI) of these products (Fig. 3, *peaks 2* and *3*) were *m/z* 317 ($M-H^+$) and *m/z* 207 ($M-H^+$), respectively. None of these metabolites were observed when arachidonic acid or (12*S*)-HPETE were administered to lysate from *E. coli* transformed with the empty vector pQE30 not containing the LOX cDNA. In order to confirm arachidonic acid as a precursor of these newly formed metabolites, labeled [$^2H_8$]arachidonic acid was administered, resulting in mass shifts of Δ+8 (Fig. 3, *peak 1*), Δ+8 (Fig. 3, *peak 2*), and Δ+6 (Fig. 3, *peak 3*) (Table II). Treatment with $NaBH_4$ shifts the λ_{max} of (Fig. 3, *peak 2*) and (Fig. 3, *peak 3*) from 279 to 237 nm. The spectroscopic data suggested a keto fatty acid for (Fig. 3, *peak 2*), which could be confirmed as (5*Z*,8*Z*,10*E*,14*Z*)-12-oxo-eicosatetraenoic acid (12-KETE) by comparison with a commercially available standard. MS data, retention time (rt), and UV of Fig. 3, *peak 3* were in accordance with the shorter chain ω-oxo acid (5*Z*,8*Z*,10*E*)-12-oxo-dodecatrieonic acid (12-ODTE) (25) previously found in moss preparations (16). 12-ODTE could arise from the cleavage of the intermediate 12-HPETE catalyzed by a lyase activity. In wounded *P. patens*, C_8 fragments were found that could be attributed to such an activity, and indeed also in transformations with the cloned LOX corresponding metabolites were detected. GC/MS after SPME or MSTFA derivatization gave (2*Z*)-octen-1-ol and 1-octen-3-ol or the respective derivatives. These products were identified by retention time, fragmentation pattern, and identity with commercially available standards. (2*Z*)- [$^2H_2$]Octen-1-ol and

```
                    *        20         *        40         *        60
PpLOX1   : MMLNRLETGSLASAFRGSREEFVVSRGRSPGRSQSIQVRASSGGDPAGWLQTASKQLGKL :  60
AtLOX3   : -----MALAKELMGYPLITERSSLVSSASHFKKRTQSTQFSINPFDRRPRKTKSGVVAAI :  55
POTLX-2  : -------------------------------------------------------MLGQI :   5

                    *        80         *       100         *       120
PpLOX1   : SSFGEK--R--K-TATSTSTRGPSGDNVQYTGVATTMKKLKVLDLIDRVADIQDDTSEIV : 115
AtLOX3   : SEDLVKTLRFSTTTGDRKSEEEEKAAVKFKVRAVVTVRNKNKEDLKETLVKHLDAFADKI : 115
POTLX-2  : VGGLIG--------GHHDSK---------KVKGTVVMMKKNALDFTDLAGSLTDKIFEAL :  48

                    *       140         *       160         *       180
PpLOX1   : GGKRVTVQLVS-KDVDPKTGES-MKSSEVIFPNWA-GLEGPAASLIDFVLEFTVPKSFGV : 172
AtLOX3   : G-RNIVLELIS-TQLDPKTKLP-KKSNAAVLKDWS-KKSKTKAERVHYTAEFTVDAAFGS : 171
POTLX-2  : G-QKVSFQLISSVQSDPANGLQGKHSNPAYLENFLFTLTPLAAGETAFGVTFDWNEEFGV : 107

                    *       200         *       220         *       240
PpLOX1   : PGAILVKNAHPNEFLLVSFELELHD-KSKAHYVTNSWVYN-TEKTGARIFFQNTAYLPDE : 230
AtLOX3   : PGAITVMNKHQKEFFLESITIEGFA-LGPVHFPCNSWVQSQKDHPDKRIFFTNQPYLPNE : 230
POTLX-2  : PGAFIIKNTHINEFFLKSLTLEDVPNHGKVHFVCNSWVYPSFRYKSDRIFFANQPYLPSE : 167

                    *       260         *       280         *       300
PpLOX1   : TPASLKALREQELINLRGDGTGERQIGDRIYDYAVYNDLGNIEQNEKFERPNLGGNDMYH : 290
AtLOX3   : TPSGLRVLREKELKNLRGDGSGVRKLSDRIYDFDVYNDLGNPDKSSELSRPKLGG-KEVP : 289
POTLX-2  : TPELLRKYRENELLTLRGDGTGKREAWDRIYDYDVYNDLGNPDQGKENVRTTLGGSADYP : 227

                    *       320         *       340         *       360
PpLOX1   : FPRRMRTGRRNTTVEAKKFPGMVYETRKTKGDFYIPRDEAFERAKMSDFLADGFRSIGHS : 350
AtLOX3   : YPRRCRTGRQSTVSDKDAESRVEKPLP-----MYVPRDEQFEESKQDTFAAGRLKAVLHH : 344
POTLX-2  : YPRRGRTGRPPTRTDPKSESRIPLILSL---DIYVPRDERFGHLKMSDFLTYALKSIVQF : 284

                    *       380         *       400         *       420
PpLOX1   : VSSKVTGFVT-RKQEFDTVEEIKKLYAKKG-EKVGGINNVLPDKEDIPEQEQYPLVFLQE : 408
AtLOX3   : LIPSLKASI--VAEDFADFGEIDRLYKEGL-LPKLGFQDDIFKKFPLP-------KVVVD : 394
POTLX-2  : ILPELHALFDGTPNEFDSFEDVLRLYEGGIRLPQGPLFKALTDAIPL--------EMIRE : 336

                    *       440         *       460         *       480
PpLOX1   : VLKPDGKMEHPLLYPLPQLLQADDTSWRSNDEFAREFLAGLNPVMITRVK-FPIRSSLDP : 467
AtLOX3   : TLQEST--KGLLKYDTPKILSKDKNAWLRDDEFARQAIAGINPVNIERVKTFPPVSNLDP : 452
POTLX-2  : LLRTDG--EGILRFPTPLVIKDSKTAWRTDEEFAREMLAGVNPIIISRLQEFPPKSKLDP : 394

                    *       500         *       520         *       540
PpLOX1   : AEFGDPTSAITKDHIEGSLEGLSVEEAVTSNRLFVVDYHDAFLPFVAKINAQQNSATYAT : 527
AtLOX3   : KIYGPQHSALTDDHIIGHLDGFSVQQALEENRLYMLDYHDIFLPFLDRINALDGRKAYAT : 512
POTLX-2  : EAYGNQNSTITAEHIEDKLDGLTVDEAMNNNKLFILNHHDVLIPYLRRINTTT-TKTYAS : 453

                    *       560         *       580         *       600
PpLOX1   : RTLLFLSKDGILKLLAIELALP-PKTVGEERITRVLTTRKDDQLWKVNWEWELAKAHVSN : 586
AtLOX3   : RTIFFLTRLGTLKPVAIELSLP-PHGPKH-RSKRVLTPPVDA---TSNWMWQLAKAHVSS : 567
POTLX-2  : RTLLFLQDNGSLKPLAIELSLPHPDGDQFGVTSKVYTPSDQG---VESSIWQLAKAYVAV : 510

             bo       li    li
                    *       620         *       640         *       660
PpLOX1   : NLITAHQVFSHFSRCHAVTEAVIICSNRNLSKLHPLMQLLAPHFKSTLEINRQARATLIA : 646
AtLOX3   : NLAGVHQLVNHWLRTHACLEPFILAAHRQLSAMHPIFKLLDPHMRYTLEINALARQSLIS : 627
POTLX-2  : NLSGVHQLISHWLNTHAVIEPFVIATNRQLSVLHPIHKLLYPHFRDTMNINAMARQILIN : 570

                  sl
                    *       680         *       700         *       720
PpLOX1   : AGGSIETHFTTRAYSLEMAAVNYKDTWTFESQALPTDLVARGMAVPDPDSPHGVRLVVED : 706
AtLOX3   : ADGVIEGGFTAGAYGMEMSAAAYKSSWRFDMEGLPADLIRRGMAIPDATQPHGLKLLIED : 687
POTLX-2  : AGGVLESTVFPSKFAMEMSAVVYKD-WVFPDQALPADLVKRGVAVEDSSSPHGVRLLIED : 629

                    *       740         *       760         *       780
PpLOX1   : YPYAADGLELWGALKAWHKEYVDIYYKDDAAVLQDSELMTWWTEMREKAHEDKKDSHGWP : 766
AtLOX3   : YPYANDGLLLWSAIQTWVRTYVERYYPNPNLIKTDSELQSWYSESINVGHADLRDADWWP : 747
POTLX-2  : YPYAVDGLEIWSAIKSWVTDYCSFYYGSDEEILKDNELQAWWKELREVGHGDKKNEPWWP : 689

                                 li   li         ho
                    *       800         *       820         *       840
PpLOX1   : ELNSKEALVDILTTVIWIPSCLHAAVNFGQYDFAGFMPHHPTLTRRLLPEHGNEKDKADF : 826
AtLOX3   : ELSTVDDLVSILTTLIWLASAQHAALNFGQYPYGGYVPNRPPLMRRLIPDESDP-EYASF : 806
POTLX-2  : EMETPQELIDSCTTIIWIASALHAAVNFGQYPYAGYLPNRPTVSRRFMPEPGTP-EYEEL : 748

                    *       860         *       880         *       900
PpLOX1   : NKNPEKYYLTSISNIDSTTTAMSVYEVLSAHCPIEEYIGERRGN--WTNNEKVLAAFKGF : 884
AtLOX3   : ISHPEKYYFSSMPSLAQTSKFMAVVDTLSTHSPDEEYIGERQQPSIWTGDAEIVEAFYGF : 866
POTLX-2  : KKNPDKAFLKTITAQLQTLLGVSLIEILSRHTTDEIYLGQRESP-EWTKDKEPLAAFDKF : 807

                                                                    li
                    *       920         *       940         *
PpLOX1   : KESVNEADAVMRARNADPKLRNRGGPVKMPYQLLRPHSKPGVTSMGVPNSITI : 937
AtLOX3   : AAEIGRIEKEIEKRNADPDRRNRCGAGVLPYELLVPSSEPGVTCRGVPNSVSI : 919
POTLX-2  : GKKLTDIEKQIIQRNGDNILINRSGPVNAPYTLLFPTSEGGLTGKGIPNSVSI : 860
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FIG. 1. **Amino acid sequence alignment of PpLOX1 in comparison with the 13-LOX AtLOX3 (GenBank™ accession no. AAF79461) from *A. thaliana* and the 9-LOX POTLX-2 from *Solanum tuberosum* (GenBank™ accession no. AAB67860).** For alignment, the ClustalX program (31) was used. The conserved amino acids are in *gray* as well as *black* background. The following conserved amino acids are *framed*: the residues involved in iron ligation (three histidines, an aspargine, and an isoleucine; *li*), and the three determinants for substrate and regiospecificity (*bo*, according to Borngraber *et al.* (36), *sl*, according to Sloane *et al.* (37), *ho*, according to Hornung *et al.* (38)). The restriction sites for signal peptidases derived with the ChloroP program (39) are marked with *plumb lines*.

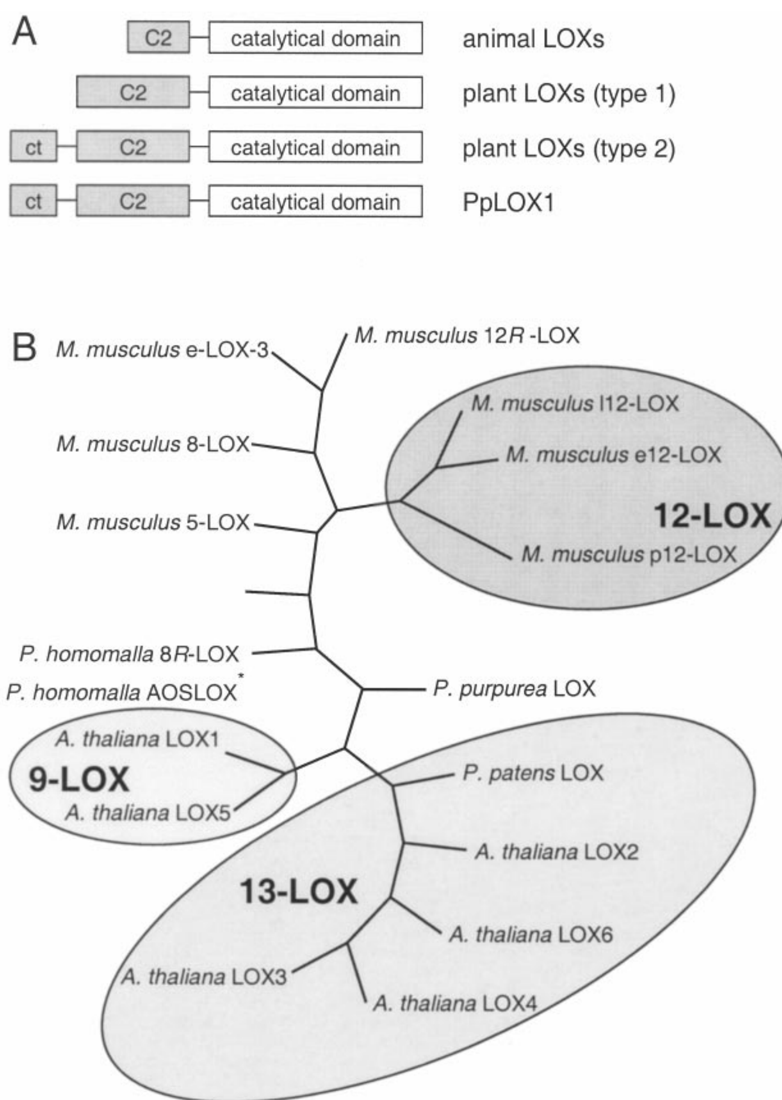

FIG. 2. **Analysis of the PpLOX1 sequence.** *A*, scheme of the domain structure of LOX from animals, plants, and PpLOX1 (*C2*, N-terminal C2 domain; *ct*, chloroplast transit peptide). *B*, phylogenetic tree analysis of PpLOX1 and selective LOX (*A. thaliana*: AtLOX1 (GenBank™ accession no. Q06327), AtLOX2 (GenBank™ accession no. P38418), AtLOX3 (GenBank™ accession no. AAF79461), AtLOX4 (GenBank™ accession no. AAF21176), AtLOX5 (GenBank™ accession no. CAC19365), AtLOX6 (GenBank™ accession no. AAG52309); *Mus musculus*: 5-LOX (GenBank™ accession no. AAC37673), 8-LOX (GenBank™ accession no. CAA75003), 12R-LOX (GenBank™ accession no. CAA74714), l12-LOX (GenBank™ accession no. AAA20658), p12-LOX (GenBank™ accession no. AAA20659), e-LOX-3 (GenBank™ accession no. CAB46101), e12-LOX (GenBank™ accession no. ACC52869); *Plexaura homomalla*: AOSLOX* (GenBank™ accession no. AAC47743), 8R-LOX (GenBank™ accession no. AAC47283); *Porphyra purpurea*: LOX (GenBank™ accession no. AAA61791)). *, the alignment was performed without the AOS domain of the *P. homomalla* AOSLOX.

TABLE I
Hydroperoxides formed from the reaction of PpLOX1 with different fatty acids at pH 8.2

In the first subrow the relative amounts of the positional isomers are given as molar ratios, the major positional isomer is printed in bold letters. In the second subrow, the relative amounts of *S*-enantiomers are given. In all cases except 6- and 9-γ-HOTE the *S*-enantiomers were the major products. All values were determined by SP-HPLC after reduction of the hydroperoxy fatty acids to their corresponding alcohols.

Substrate	Products				
Arachidonic acid	15-HETE	**12-HETE**	11-HETE	8-HETE	5-HETE
Total hydroperoxides (%)	4 ± 1.4	**90 ± 24.3**	1 ± 0	3 ± 1.4	1 ± 0
S-Enantiomer (%)	52 ± 4	**97 ± 3**	49 ± 2	43 ± 7	51 ± 28
Eicosapentaenoic acid	**12-HEPE**	8-HEPE			
Total hydroperoxides (%)	**97 ± 16.6**	3 ± 1.4			
S-Enantiomer (%)	**99 ± 0**	48 ± 28			
Docosahexaenoic acid	**14-HDHE**	10-HDHE			
Total hydroperoxides (%)	**96 ± 3**	4 ± 3			
S-Enantiomer (%)	**99 ± 1**				
γ-Linolenic acid	13-γHOTE	**10-γHOTE**	9-γHOTE	6-γHOTE	
Total hydroperoxides (%)	15 ± 1.4	**76 ± 8.1**	3 ± 0	7 ± 5.4	
% *S*-Enantiomer (%)	78 ± 4	**98 ± 1**	39 ± 2	36 ± 7	
α-Linolenic acid	16-HOTE	**13-HOTE**	12-HOTE	9-HOTE	
Total hydroperoxides (%)	6 ± 3.7	**71 ± 9.8**	6 ± 2.5	17 ± 3.7	
S-Enantiomer (%)		**91 ± 7**		61 ± 25	
Linoleic acid	**13-HODE**	9-HODE			
Total hydroperoxides (%)	**83 ± 13.4**	17 ± 12.2			
S-Enantiomer (%)	**94 ± 2**	46 ± 4			

1-[$^2H_2$]octen-3-ol were detected after administration of [$^2H_8$]arachidonic acid (Table II).

The intermediate 12-HPETE was transformed nearly quantitatively to 12-KETE (Fig. 3, *peak 2*), 12-ODTE (Fig. 3, *peak 3*), and the C_8 alcohols within the first 2 min after substrate addition (Fig. 4). Arachidonic acid content decreased rapidly and was detected only until 4 min after the onset of the reaction. The enantiomeric excess of 12-HPETE remained above

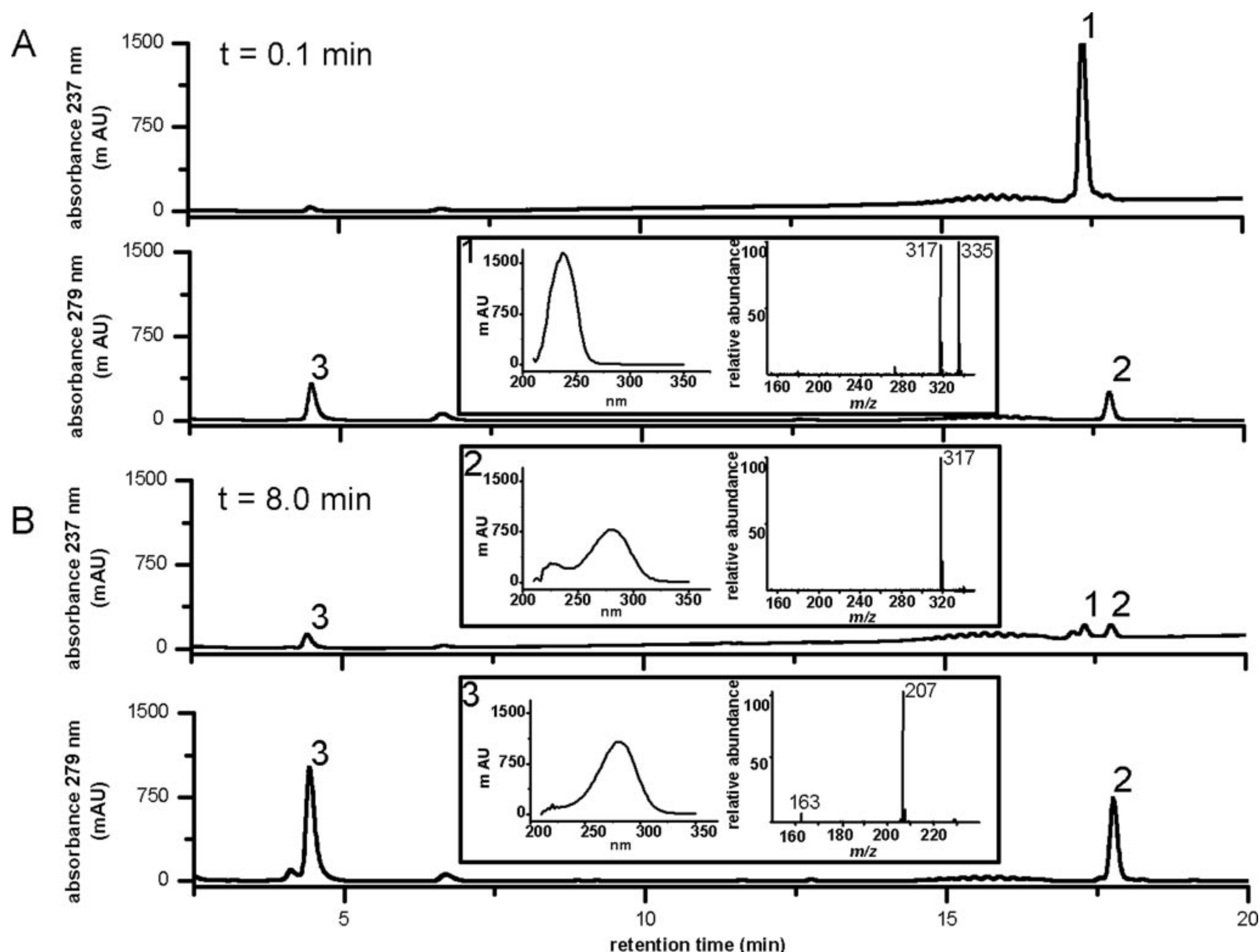

FIG. 3. **HPLC analysis of reaction products of arachidonic acid formed by PpLOX1-preparations.** *A*, 0.1 min and *B*, 8 min after the addition of arachidonic acid. Numbers indicate the resulting PpLOX1 products: 1,12-HPETE; 2,12-KETE; 3,12-ODTE. The *insets* show the respective UV- and negative ion ESI-MS spectra of the products.

98% (*S*) during the observed time course from 20 s to 8 min of the reaction indicating that *S*-enantiomers are substrates for the hydroperoxidase activity.

Transformation of eicosapentaenoic acid, docosahexaenoic acid, and γ-linolenic acid leads to the formation of the ω-oxo-acids 12-ODTE, (4*Z*,7*Z*,10*Z*,12*E*)-14-oxo-tetradecatetraeonic (14-ODTE), and (6*Z*,8*E*)-10-oxo-decadieonic acid (10-ODDE), respectively (Tables II and III). 14-ODTE and 10-ODDE were identified by ESI/MS (m/z = 233 (M-H^+), m/z = 189 (M-44), and m/z = 181 (M-H^+), m/z = 137 (M-44), respectively). The ω-oxo acids exhibited UV maxima at λ_{max} = 282 nm. All transformations resulted in C_8 alcohols as second cleavage product. Eicosapentaenoic acid and docosahexaenoic acid gave (2, 5)-octadien-1-ol and (1, 5)-octadien-3-ol as second fragments, which were identified by GC/MS and confirmed by HR-GC/MS after derivatization with MSTFA ($[M-15]^+$ calculated for $C_{10}H_{19}OSi$: 183.1205, found 183.1212 [(2,5)-octadien-1-ol] and 183.1214 [(1, 5)-octadien-3-ol]). (2*Z*)-Octen-1-ol and 1-octen-3-ol were formed from γ-linolenic acid. A significant lyase activity of PpLOX1 was only observed, if intermediates with the hydroperoxy function at position ω-9 were formed and, consequently, if unsaturated C8-alcohols could be liberated. None of the other isomeric hydroperoxides derived from the above fatty acids were transformed to detectable amounts of lyase products. Moreover, PpLOX1 exhibited no major lyase activity if α-linolenic acid or linoleic acid were administered as substrates. Keto acid formation was observed in all transformations (Table III).

DISCUSSION

Based on sequence similarities to higher plants, we were able to identify a LOX from an EST library of the moss *P. patens*. We cloned and overexpressed this LOX, which allowed a functional characterization. The present study is to our knowledge the first to describe a LOX/lyase/hydroperoxidase activity within a single cloned enzyme. Our results support the findings obtained with crude preparations or partially purified LOX that these enzymes have a multiple potential to transform hydroperoxide fatty acids (8, 28, 29). The PpLOX has a relaxed substrate tolerance and exhibits a remarkable diverse product spectrum. Keto acids, hydroperoxides, and their cleavage products could be detected from this single enzyme. This lipoxygenase can thus account for the observed fatty acid hydroperoxides and at least three different lyase products detected previously in the moss. High flexibility and diverse product production of one single enzyme have been described previously for terpene synthases, enzymes from phenylpropanoid metabolism and from polyketide biosynthesis (30), but this report is the first to prove a diverse product spectrum of a single LOX.

Sequence analysis identified PpLOX, which shows high homologies to an *A. thaliana* linoleate 13-LOX (Fig. 1) as a member of the plant type2-13-LOX-subfamily (Fig. 2*B*). Although PpLOX1 shows highest homologies to plant type2-LOX, it exhibits a substrate- and regiospecificity, which is more typical

TABLE II
Analytical data for the major products of lyase and hydroperoxidase activity

Compound	rt[a]	λ_{max}[b]	*m/z*-values of diagnostic ions
	min	*nm*	*method; m/z intensity*
Oxo acids			
12-ODTE[c]	4.48	282	Neg ESI; 207 (M-H$^+$100%), 163 (5%)
12-[$^2H_6$]ODTE	4.23	282	Pos APCI; 215 (M+H$^+$100%), 197 (30%)
14-ODTE	6.57	282	Neg ESI; 233 (M-H$^+$100%), 189 (10%)
10-ODDE	3.12	282	Neg ESI; 181 (M-H$^+$100%), 137 (15%)
Keto acids			
12-KETE[c]	17.64	282	Neg ESI; 317 (M-H$^+$100%)
12-[$^2H_7$]KETE	17.43	282	Neg ESI; 324 (M-H$^+$100%)
12-KEPE	15.64	282	Neg ESI; 315 (M-H$^+$100%)
14-KDHE	17.10	282	Neg ESI; 341 (M-H$^+$100%)
10-γKOTE	16.02	282	Neg ESI; 291 (M-H$^+$100%)

Alcohols	SPME-GC/MS	HR-MS of Me_3Si derivative
	m/z	*m/z*
2-Octen-1-ol[c]	110 [M-18]$^+$	[M-15]$^+$: 185.1356 (-2.9 ppm)
1-Octen-3-ol[c]	110 [M-18]$^+$	[M-15]$^+$: 185.1367 (-3.0 ppm)
2-[$^2H_2$]Octen-1-ol	112 [M-18]$^+$	ND[d]
1-[$^2H_2$]Octen-3-ol	112 [M-18]$^+$	ND
(2,5)-Octadien-1-ol	ND[d]	[M-15]$^+$: 183.1212 (3.7 ppm)
(1,5)-Octadien-3-ol	ND	[M-15]$^+$: 183.1214 (4.8 ppm)

[a] A nucleosil C18 column (ODS-3 125 mm x 2 mm) was used with a 20 min gradient from 45% aqueous acetonitrile (0.5% HAc) to 100% acetonitrile (0.5% HAc) at a flow rate of 0.2 ml min^{-1}.
[b] UV spectra recorded in aqueous acetonitrile during the HPLC run.
[c] Compounds were identical with commercial or synthetic standards.
[d] ND, not determined.

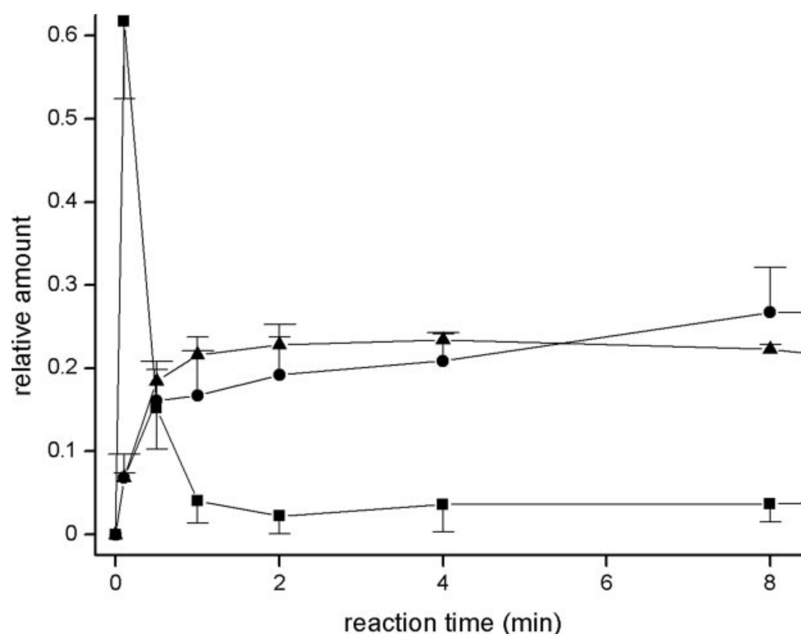

FIG. 4. **Time course of product formation from 0.5 mM arachidonic acid by PpLOX1 (*n* = 3).** ■, 12-HPETE, ●, 12-ODTE; ▲, 12-KETE. UV absorptions were normalized with the extinction coefficient. The *error bars* are based on S.D.

for animal and algal enzymes. While plant type2-LOX transform polyunsaturated C_{18} fatty acids to the corresponding 13-hydroperoxides and arachidonic acid to the corresponding 15-hydroperoxides (31), PpLOX1 converts C_{20} fatty acids as preferred substrates to the 12-hydroperoxides. This unusual activity might reflect the different phylogenetic history of mosses, algae and higher plants. Plant type2-LOX are commonly targeted to chloroplasts. The PpLOX1-sequence harbors this plastidial targeting signal as well, implying that the enzyme is located in plastids (Fig. 2*A*).

Detailed biochemical analysis revealed that the PpLOX1 has a high substrate tolerance and can metabolize fatty acids with chain lengths from 18 to 22 carbons, which results in hydroperoxy fatty acids with different positional isomers (Table I). The regiospecificity for the introduction of dioxygen by many LOX is determined by the distance from the terminus of the fatty acids (31). In the case of PpLOX1, the ω-9 position was attacked preferentially. Transformation of C_{20} and C_{22} fatty acids occurred with high regiochemistry at the ω-9-position, γ-linolenic acid was transformed with significantly lower specificity (Table I). 10-γHOTE was found in only 76% besides 13-, 9-, and 6-γHOTE. Only with α-linolenic acid and linoleic acid, where the ω-9 position cannot be attacked, ω-6 hydroperoxides are the major products. While PpLOX1 is generally specific for the production of *S*-hydroperoxides the transformation products 9 and 6-γHOTE were predominantly *R*-enantiomers. This can be explained with a model where γ-linolenic acid is not completely fixed in the enzymatic pocket but a rather flexible attack at different positions of the pentadiene system can occur.

From kinetic data and the regio- and stereospecificity of prod-

TABLE III

Major products formed from the reaction of PpLOX1 with different fatty acids

A

Substrate	Major products obtained by lipoxygenase-activity	Products obtained by lyase-activity	Keto fatty acids
Arachidonic acid (20:4, n-6)	HOO, COOH	OH [1,2,5]; OH [1,2,5]; O, COOH [4,5]	12-KETE 15-KETE
Eicosapentaenoic acid (20:5, n-3)	HOO, COOH	OH [2,3]; OH [2,3]; O, COOH [4]	12-KEPE

B

Substrate	Major products obtained by lipoxygenase-activity	Products obtained by lyase-activity	Keto fatty acids
Docosahexaenoic acid (22:6, n-3)	HOO, COOH	OH [2,3]; OH [2,3]; O, COOH [4]	14-KDHE
γ-Linolenic acid (18:3, n-6)	HOO, COOH	OH [1,2]; OH [1,2]; O, COOH [4]	10-γKOTE 13-γKOTE 6-γKOTE
α-Linolenic acid (18:3, n-3)	HOO, COOH	**No major lyase products**[6]	9-αKOTE 13-αKOTE
Linoleic acid (18:2, n-6)	HOO, COOH	**No major lyase products**	13-KODE 9-KODE

The lyase products were identified by:

[1] SPME and GC/MS (EI) compared to commercial or synthetic standards.

[2] MSTFA-derivatization and GC/MS (EI).

[3] MSTFA-derivatization and HR-MS (EI).

[4] RP-HPLC/MS (ESI).

[5] The deuterated products were detected after administration of [$^2H_8$]arachidonic acid.

[6] Using sensitive GC/MS analysis (40), traces of putative (9*Z*,11*E*)-13-oxo-trideca-9,11-dienoic acid were detected. Keto acids are sorted according to their relative abundance.

uct formation, it can be concluded that the C_{20} fatty acids arachidonic acid and eicosapentaenoic acid are the preferred substrates for PpLOX1. This substrate preference is in accordance with previous findings on crude tissue preparations of this moss, where arachidonic acid was identified as precursor of structurally diverse unsaturated aldehydes and alcohols (16). In contrast to higher plants, mosses contain large amounts of polyunsaturated C_{20} fatty acids, which might have led to this observed preference. As in the transformations with PpLOX1, the tissue extracts discriminated for free fatty acids, while methyl esters were not accepted as substrates for aldehydes and alcohol formation (16). Using derivatization and GC/MS we identified predominantly the 2*Z* isomer of octen-1-ol, whereas 2*E*-octen-1-ol was reported from the moss. Careful reinvestigation showed that the moss also releases elevated amounts of the 2*Z* isomer, which was poorly resolved in previous analyses (16).

Remarkably, the PpLOX1 is a multifunctional enzyme. Besides hydroperoxide formation PpLOX1 exhibited pronounced lyase activity, releasing ω-oxo acids and unsaturated short chain alcohols. In addition, the corresponding keto acids were formed. That these fragments are of fatty acid origin could be demonstrated after addition of stable isotope labeled precursors that gave rise to labeled products. The lyase activity of PpLOX1 was detected in all cases where intermediate hydroperoxy fatty acids with the *S*-hydroperoxide function at position ω-9 were formed (Table III). The PpLOX1 has an unusual variable aptitude to generate ω-oxo acids with chain length of 10–14 carbons. In the case of other positional isomers of hydroperoxy fatty acids no significant lyase activity, but hydroperoxidase activity was detected (Table II). Most remark-

ably the lyase activity differs from that of a *Vicia sativa* LOX where linoleic acid transformation to 2,4-decadienal occurs not via the hydroperoxides, but, moreover, with a peroxyl radical as intermediate (29). It also differs from the 1-octen-3-ol forming activity in mushrooms, which is based on two separate enzymes with LOX and HPL activity, respectively (19).

When arachidonic acid was added as a substrate, the intermediate 12-HPETE was transformed nearly quantitatively within the first minute after substrate addition to yield 12-KETE, 12-ODTE, (2*Z*)-octen-1-ol, and 1-octen-3-ol (Figs. 3 and 4). This kinetic behavior followed similar patterns as observed after wounding of the moss itself (16). This suggests that only one single enzyme is required for the formation of several oxylipins found in wounded *P. patens* tissue. A comparable fast formation of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes has also been observed in previous investigations of the fatty acid metabolism of wounded diatoms where these metabolites act as chemical defense molecules (32). After tissue disruption a rapid onset of aldehyde production occurs in wounded diatoms and stationary levels of these metabolites are present within the first minutes after cell disruption (25). Interestingly, the freshwater diatom *Asterionella formosa* is also able to transform arachidonic acid to 12-ODTE upon wounding, but as second cleavage product (1,3*E*)-octadiene is released (2). Our findings now explain how PpLOX1 generates 12-ODTE from the same substrate. Different C_8 fragments are released by the moss, demonstrating that mosses and diatoms have evolved alternative pathways for the formation of 12-ODTE.

Only a few studies of LOX from mosses have been reported (4) and most focus on the transformation of externally applied substrates to crude preparations. It is known that other mosses also release volatile C_8 and C_9 alcohols and aldehydes, suggesting that the mechanism of eicosanoid transformation described here might be universally distributed in this phylum (13). Besides the C_8 alcohols identified here, *P. patens* also releases 2*E*-nonenal and 11-oxo-undeca-5,9-dienoic acid from arachidonic acid upon tissue disruption (16). This indicates that an additional not yet identified lyase pathway is active in the moss.

More detailed investigations of the formation of unsaturated aldehydes from fatty acid hydroperoxides have been carried out on plant or animal LOX. Few LOX with lyase activity have been described that release $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes and a second alcohol fragment from fatty acids like it was observed with the PpLOX1 (28, 29). Glasgow *et al.* (8) investigated a preparation of porcine leukocytes that converts arachidonic acid to a mixture of products derived via 5-, 12- and 15-LOX reactions and also identified 12-ODTE as a proposed cleavage product of 12-HPETE. All studies on LOX/lyases to date have been carried out with crude preparations or partially purified enzymes. Koljak *et al.* (11) identified a fused peroxidase/lipoxygenase from the coral *Plexaura homomalla* likely providing for prostanoids. In contrast to the domain structure of this enzyme, the sequence analysis of the PpLOX1 gives us no indication for a peroxidase active site. Thus the lyase activity observed is most likely not due to a fused domain structure of a LOX and a lyase but rather to a bi-functional LOX activity.

Like most $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes, 12-ODTE exhibits antiproliferative activity against fast growing and dividing cells (33, 34). The 12-ODTE producing PpLOX1 might accordingly be regarded as enzyme involved in an activated chemical defense of *P. patens* although pathogens or herbivores that might be targeted by this chemical are not identified as of yet. The assumption that PpLOX1 is involved in the chemical defense is strongly supported by the above-mentioned similarities of the defensive reactions in diatoms and mosses. Earlier it was proposed that a LOX and HPL activity is involved in the formation of defensive principles with an $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde structure and also for the octenols (2, 18, 19, 35). In the present study we show that the cloned PpLOX1 exhibits both activities required for the production of oxo acids and alcohols from polyunsaturated fatty acids. According to our finding this single LOX can account for high product diversity. The newly arising picture for the wound-activated reaction of mosses thus involves only one key enzyme required for the transformation of free fatty acids.

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Biosynthesis of C9-aldehydes in the moss *Physcomitrella patens* ☆

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Abstract

After wounding, the moss *Physcomitrella patens* emits fatty acid derived volatiles like octenal, octenols and (2*E*)-nonenal. Flowering plants produce nonenal from C18-fatty acids via lipoxygenase and hydroperoxide lyase reactions, but the moss exploits the C20 precursor arachidonic acid for the formation of these oxylipins. We describe the isolation of the first cDNA (*PpHPL*) encoding a hydroperoxide lyase from a lower eukaryotic organism. The physiological pathway allocation and characterization of a downstream enal-isomerase gives a new picture for the formation of fatty acid derived volatiles from lower plants. Expression of a fusion protein with a yellow fluorescent protein in moss protoplasts showed that PpHPL was found in clusters in membranes of plastids. PpHPL can be classified as an unspecific hydroperoxide lyase having a substrate preference for 9-hydroperoxides of C18-fatty acids but also the predominant substrate 12-hydroperoxy arachidonic acid is accepted. Feeding experiments using arachidonic acid show an increase in the 12-hydroperoxide being metabolized to C8-aldehydes/alcohols and (3*Z*)-nonenal, which is rapidly isomerized to (2*E*)-nonenal. PpHPL knock out lines failed to emit (2*E*)-nonenal while formation of C8-volatiles was not affected indicating that in contrast to flowering plants, PpHPL is only involved in formation of a specific subset of volatiles.
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Keywords: Nonenal; Cytochrome P-450; Lipid peroxidation; Oxylipin metabolism; Moss; Substrate specificity

1. Introduction

Oxylipin is a collective term for oxygenated metabolites derived from polyunsaturated fatty acids (PUFAs). Most oxylipins are bioactive compounds involved in signal and defense reactions in mammals, higher plants and algae [1–3].

Abbreviations: AOS, allene oxide synthase; DES, divinyl ether synthase; DNPH, 2,4-dinitrophenylhydrazin; H(P)ETE, hydro(pero)xy-arachidonic acid; HPL, hydroperoxide lyase; H(P)ODE, hydro(pero)xy-linoleic acid; H(P)OTE, hydro(pero)xy-linolenic acid; LOX, lipoxygenase

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession number CAC86920.

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The biosynthesis of many oxylipins is initiated by the conversion of PUFAs by a lipoxygenase (LOX) [4]. While in flowering plants C18-fatty acids like linoleic (18:2) and α-linolenic acid (α-18:3) are the main precursors of such compounds, in animals and algae oxylipins derive predominantly from C20-fatty acids [1,2,5].

In plants LOX-derived hydroperoxides serve as substrates for at least 7 enzyme families, including three subfamilies of *CYP74*-type of cytochrome P-450: hydroperoxide lyase (HPL), allene oxide synthase (AOS) and divinyl ether synthase (DES) [6]. In contrast to typical P-450 monooxygenases, *CYP74*-enzymes require neither O_2 nor NADPH as cofactors [4]. Instead, the hydroperoxide group of the fatty acid substrate serves as oxygen donor and as a source of reducing equivalents.

By trapping experiments Grechkin and Hamberg [7] have recently found that HPL catalyzes the isomerization of fatty acid hydroperoxide into an unstable hemiacetal, which is spontaneously

decomposed into a short chain aldehyde and the corresponding ω-oxo fatty acid [7], whereas Noordermeer and co-workers [8] have proposed the formation of an allylic ether cation. In contrast, the AOS converts hydroperoxides to unstable allene oxides, which are hydrolyzed into α- and γ-ketols or can spontaneously undergo non-enzymatic cyclization [9]. In the presence of an allene oxide cyclase, the allene oxide is metabolized to 12-oxo phytodienoic acid. Subsequent reduction of 12-oxo phytodienoic acid by a reductase and three cycles of β-oxidation yields jasmonic acid [9]. DES produces divinyl ether which can be cleaved into short chain aldehydes and ω-oxo fatty acids under acidic conditions [10]. Several *CYP74*-enzymes have been cloned from different plant species. Some of them contain plastidic transit peptides, for instance an AOS from *Arabidopsis thaliana* [11]. A plastidic association was shown for the AOS and the HPL from tomato and for the AOS from barley [12,13].

In flowering plants, the basic substrates for *CYP74*-enzymes are 13- or 9-hydroperoxides of linoleic or α-linolenic acid. Based on their substrate specificity the enzymes may thus be classified into 13- or 9-hydroperoxide-specific enzymes, or unspecific enzymes. The short chain aldehydes of the HPL metabolization of 13-hydroperoxy linoleic (13-HPODE) or 13-hydroperoxy α-linolenic acid (13-HPOTE) are hexanal and (3*Z*)-hexenal, respectively [14]. The second resulting fragment, a C12 ω-oxo fatty acid may isomerize to traumatin, a wound hormone [15]. The volatile reaction products of 9-hydroperoxides (9-HPODE or 9-HPOTE) are (3*Z*)-nonenal and (3*Z*,6*Z*)-nonadienal, respectively. As in the case of traumatin the unsaturated (3*Z*)-aldehydes can further be metabolized by an isomerase into the (2*E*)-enal isoforms [16].

Short chain aldehydes are components of the green odor of plants and have an antimicrobial effect in vitro. The physiological function of aldehydes is mainly addressed to plant defense against microbes and herbivores [3,17]. This effect has, for example, been demonstrated upon inoculation of French bean with an avirulent variety of *Pseudomonas syringae* pv. *syringae*. Whereas this treatment resulted in production of C6-aldehydes, a virulent strain did not lead to such an increase [18]. Different C6-volatiles have also been shown to induce defense related genes and to stimulate phytoalexin accumulation [19,20]. In transgenic potato plants lacking *HPL* transcript an increase in aphid performance was observed [21]. Recently, the antimicrobial effect of C9-volatiles has been described as well [22].

In contrast to other eukaryotic organisms, the knowledge on the biosynthesis and function of oxylipins in mosses is limited. These organisms contain C18, C20, and longer chain length fatty acids [23]. In the moss *Physcomitrella patens*, for example, the main fatty acids are palmitic acid, linoleic acid and arachidonic acid (20:4) [24]. Certain mosses are known to emit aldehydes and their corresponding alcohols, like octenal, octenol but also hexanal and hexenal [23]. Recently, it was shown that the moss *P. patens* produces oct-1-en-3-ol, oct-2-en-1-ol and (2*E*)-nonenal after wounding. These volatiles are all derived from arachidonic acid [25]. While the octenols may be produced by the action of a novel LOX with a lyase activity that has been described recently [26], the enzyme responsible for C9-volatile production is still unknown.

Here, we present an in depth investigation of the second line of transformation of arachidonic acid hydroperoxides in *P. patens*. We introduce the isolation, characterization and localization of a new HPL from the moss and elucidate the further fate of the HPL products. The substrate specificity against hydroperoxides of different fatty acids was analyzed and we show that the enzyme is localized at a inner membrane of chloroplasts. Using a knock-out mutant for this HPL, we demonstrate that the release of (2*E*)-nonenal after wounding is mediated by this enzyme and is derived from 12-hydroperoxy arachidonic acid (12-HPETE).

2. Material and methods

2.1. Isolation, expression and purification of recombinant PpHPL

An EST-library from *P. patens* was provided by BASF Plant Science, and a partial EST-clone with a putative *CYP74*-sequence was identified based on sequence similarity to known *CYP74*-sequences from flowering plants. A 1470 bp PCR fragment was amplified with the sense primer 5′-CGT ACG GTT GTA GCC AGT CTT GGG-3′ and the antisense primer 5′-TCA ATC TGA TCG CGG CGT CAG TG-3′ using the partial EST-clone as a template. A ^{32}P-labeled cDNA probe of the PCR fragment was synthesized using HexaLabel DNA Labeling Kit from Fermentas and used to screen a lambda ZAPExpress cDNA library of moss gametophytes. The longest insert (*PpHPL*) was sequenced and used as a template in a PCR-based approach to construct a vector for recombinant expression of PpHPL: Forward primer (5′-GGA TCC ATG GAT CGC ACT TTA GTT C-3′) and reverse primer (5′-AAG CTT TCA ATC TGA TCG CGG CGT CAG TG-3′) for the amplification introduced *Bam*HI and *Hin*dIII restriction sites, respectively. To express a truncated protein, starting at amino acid 44, a primer was designed that introduced a *Bam*HI restriction site. Using these restriction sites it was possible to clone *PpHPL* and the truncated cDNA in-frame into the pQE30 expression vector. For heterologous expression, pQE30-PpHPLfull and pQE30-PpHPLtrunc was transformed into *E. coli* host strain SG13009 pREP4. Purification of recombinant PpHPL was performed as described previously [27].

2.2. Product Analysis of recombinant PpHPL

To produce fatty acid hydroperoxides, the corresponding fatty acids were incubated with recombinant cucumber lipid body LOX (production of 15-, 12-, 8-HPETE, 13-HPOD/TE, 13-γHPOTE) or potato tuber LOX (production of 11-, 5-HPETE, 9-HPOD/TE, 9-γHPOTE) [28]. Labeled fatty acid hydroperoxides were prepared by incubation of [1-^{14}C]-linoleic acid (Perkin Elmer Life Science, Boston, MA, USA; specific activity 2.0 GBq/mmol) with either soybean 13-LOX (Sigma, Germany) or recombinant potato tuber LOX [28]. Radiolabeled fatty acid was diluted with unlabeled linoleic acid in a mol-ratio 1:50.

For product analysis, recombinant PpHPL as well as 13-AOS (CAD29735), 9-AOS (CAI30876), 13-DES (CAI30435) and 9-DES (CAC28152), diluted in 2 ml of 100 mM sodium phosphate buffer was incubated with either 100 nmol [1-^{14}C]-13-HPODE or [1-^{14}C]-9-HPODE for 30 min at room temperature. The reaction was stopped by adding 40 μl of glacial acetic acid and extracted with methanol/chloroform [29]. The obtained samples were dissolved in HPLC solvent and analyzed as described [30]. For determination of kinetic parameters of the recombinant PpHPL, the initial reaction velocity was determined at 12 different concentrations. For substrate specificity assay recombinant enzyme was incubated with 30 μM of each hydroperoxide, and the decrease in absorbance at 234 nm was measured spectrophotometrically.

To identify the aldehydes produced, the reaction mixture was acidified to pH 3 with HCl and incubated with 2.5 ml ethanol containing 0.1% 2,4-dinitrophenylhydrazin (DNPH) for 1 h at room temperature. Heptanal was added as an internal standard. The hydrazones were extracted twice with hexane, evaporated under a stream of nitrogen and subjected to HPLC analysis [31]. ESI/MS analyses were performed by direct injection of derivatized aldehydes into ESI interface of an LCQ ion trap mass spectrometer (Thermo Electron,

Germany). The mass spectrometer was operated in negative ion mode with the source voltage set to 4.5 kV, and a capillary voltage of 40 V and 300 °C. In full MS mode, scans were collected between *m/z* values of 150 and 500.

2.3. Transient expression of YFP fusion constructs in P. patens and microscopy

The entire cDNA without the stop-codon was amplified containing *Nco*I restriction sites at the 5′ and 3′ ends. This fragment was ligated into the *Nco*I-restricted vector pCAT_YFP [32] in order to express PpHPL as YFP-fusion protein. Plasmid DNA from this construct and from pCAT_YFP carrying no insert were transiently transfected into *P. patens* protoplasts as described previously [33]. Localization of YFP and its fusions in transfected protoplast was analyzed after 3 days of expression by confocal laser scanning microscopy according to [34].

2.4. Creating and analyzing PpHPL-knock-out mosses

A region of the PpHPL cDNA comprising the nucleotides 372 to 1710 was amplified by PCR using the following primers: 5′-TGG GGC AAG AGA GCA ATC TG-3′ (forward primer) and 5′-AAA GCT CCG CCA GAA GAA GG-3′ (reverse primer). The PCR product was cloned into the *Eco*RV restriction site of pGEM-5Zf(+) (Promega). The selection marker cassette (*nos*-promoter:: neomycin phosphotransferase::*nos*-terminator) derived from the vector pBIN19 [35] was cloned into the *Mls*I site of the *PpHPL* cDNA fragment. A resulting construct showing conformity of the selection cassette with the cDNA orientation was selected for plant transformation. Before transformation, the construct was digested with *Aat*II and *Not*I to release the disruption construct. 25 μg of DNA were used for plant transformation. The transformation of *Physcomitrella* protoplasts and selection of transgenic lines was performed according to [36]. Using gene-specific primers, transformants were screened in addition for lost of wild type HPL allele band and incorporation of the nptII cassette into the *PpHPL*-locus. Further analyses were performed with several independent transformants to exclude effects resulting from non-homologous recombination events.

2.5. Analytical methods

Moss cultures were grown under sterile conditions [37]. For determination of endogenous fatty acid, hydro(pero)xides 3 to 4 g of moss material was analyzed as described [38]. To analyze lyase products after feeding of different fatty acids, 4 ml of a suspension culture of each transformant was filtered to give 100–150 mg fresh weight biomass, which was resuspended in 1 ml fresh medium. The samples were cooled to 4 °C and treated with ultrasound for 2 min using a Labsonic L 1000 ultrasonic oscillator (B. Braun, Germany) and 200 μg of fatty acid was added. To characterize the emitted volatiles, 2 μl of internal standard (1 mM 2-decanon in methanol) was added, the vial was sealed with a Teflon septum, and a polydimethyldiloxane-coated fiber (Supelco, Germany) was inserted for 15 min in the gas phase [25]. Separation was performed on a Finnigan Trace GC/MS equipped with a 15 m EC5 column ID 0.25 m, 0.25 μm film thickness (Alltech, Deefield, Il). The temperature program started at 40 °C (4 min) and ramped to 120 °C with a rate of 5 K min^{-1}, then with 20 K min^{-1} to 280 °C (2 min).

Products were identified by comparison with commercial available (Figs. 7 and 8: 1,2,4) and synthesized (Fig. 7: 3,8) standards or tentatively assigned by

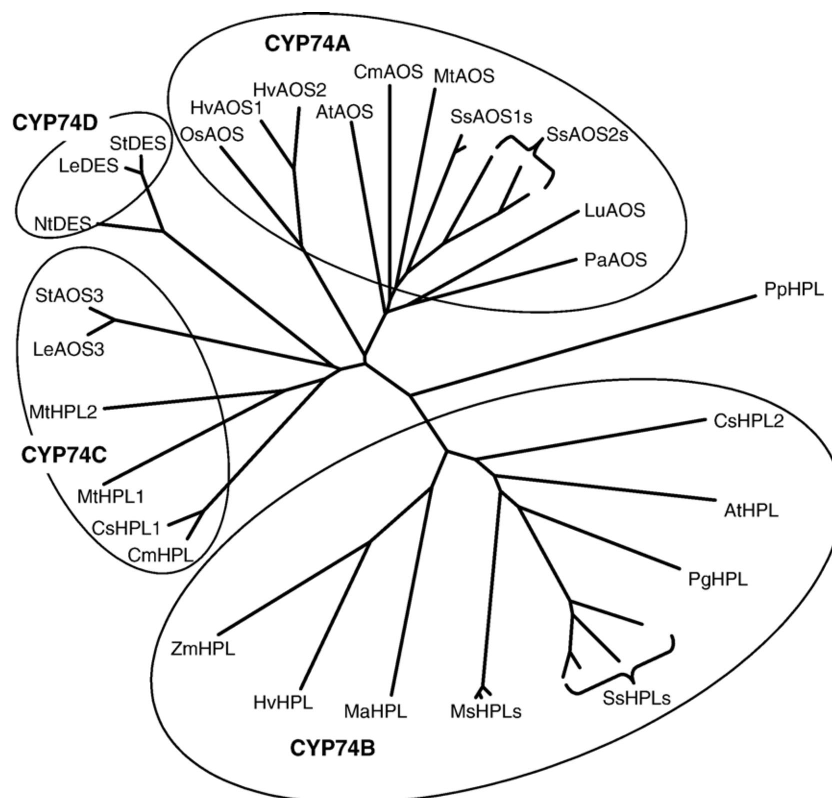

Fig. 1. Phylogenetic relationship of *CYP74* families. Amino acid sequences were aligned using ClustalX [58]. The phylogram was constructed using Treeview [59]. Based on sequence identity four *CYP74*-subfamilies (A to D) are indicated. Amino acid sequences corresponding to the following Acc.-No. were used for the analysis: *A. thaliana* (AtAOS: CAA63266, AtHPL: AAC69871), melon (CmAOS: AAM66138, CmHPL: AAK54282), cucumber (CsHPL1: AAF64041, CsHPL2: AF229812), barley (HvAOS1: CAB86384, HvAOS2: CAB86383, HvHPL: CAC82980), tomato (LeAOS3: AAN76867, LeDES: AAG42261), flax (LuAOS: AAA03353), banana (MaHPL1: CAB39331), *Medicago sativa* (MsHPLs: CAB54847, CAB54848, CAB54849), *Medicago truncatula* (MtAOS: TC22359, MtHPL1: CAC86898, MtHPL2: CAC86899), tobacco (NtDES: AAL40900), rice (OsAOS: AAL38184), guayule (PaAOS: CAA55025), *Psidium guajava* (PgHPL: AAK15070), *Physcomitrella patens* (PpHPL: CAC86920), different Solanaceae species (SsAOS1s: CAB88032, CAD29735, SsAOS2s: AAF67141, CAD29736, CAC82911, SsHPLs: AAF67142, AAA97465, CAC44040, CAC91565), potato (StAOS3: CAI30876, StDES: CAC28152), maize (ZmHPL: AAS47027).

MS-spectra (Fig. 9: 5,7) and UV absorption according to [22]. (3*Z*)-nonenal and 12-ODTE were synthesized according to [39]. For trapping of (3*Z*)-enal products and for structure elucidation of the ω-oxo fatty acids the filtered moss was resuspended in 1 ml derivatization reagent (25 mM pentafluorobenzyl hydroxylamine in 100 mM Tris/HCl, pH 7) and treated as described above after addition of 5 μl internal standard (1 mM benzaldehyde in MeOH). Extraction of oxime-derivates, subsequently derivatization treatments and analyses by GC/MS were done according to [40].

3. Results

3.1. Isolation and characterization

Previous studies suggested that the moss *P. patens* might contain a second 12-HPETE metabolizing activity besides a multifunctional arachidonate 12-LOX [26], which is involved in the formation of (2*E*)-nonenal [25]. As this conversion is rather unusual, we set out to clone the corresponding gene of a putative HPL, analyze the recombinant protein and gain insight into the fatty acid-derived aldehyde and alcohol formation in mosses. In an EST database we found a partial clone with similarity to known *CYP74*-enzymes from flowering plants. To isolate the full-length cDNA clone we screened a cDNA library of *P. patens* gametophytes. Of about 1×10^6 plaques screened, 12 positives were isolated and partially sequenced. The isolates were identical in the overlapping regions. The longest insert sequenced had a length of 1975 bp excluding its poly(A) tail. The coding sequence started at position 190 and ended at position 1599 of the isolated cDNA fragment. A stop-codon located 30 bp upstream of the start-Met indicated that the clone is full length. The deduced protein consists of 532 amino acids and has a predicted molecular mass of about 59.9 kDa. The amino acid sequence was most similar to that of AOS from *Arabidopsis thaliana* (61%; Acc.-No. CAA73184) and *Cucumis melo* (60%; Acc.-No. AAM66138). PpHPL contained typical *CYP74* motifs and amino acids within its sequence, however, the amino acid sequence did not reliable group into any existing subfamily (*CYP74*A to *CYP74*D). Currently, enzymes are grouped into subfamilies based on sequence identity higher than 55% (an arbitrary definition known from mammalian cytochrome P450 enzymes). PpHPL exhibits less than 50% amino acid identity to other *CYP74*s, and therefore cannot easily be categorized into the known subfamilies using the established criteria of sequence identity (Fig. 1). The N-terminal 39 amino acids of PpHPL were recognized as a plastidic transit peptide by the signal peptide prediction program TargetP (http://www.cbs.dtu.dk/services/).

When either the full length protein or the truncated protein lacking the plastidic transit peptide was expressed in *E. coli* only the truncated protein metabolized fatty acid hydroperoxides. Therefore, all further analyses were performed with the truncated protein. Using [1-^{14}C]-13-HPODE as well as [1-^{14}C]-9-HPODE as a substrate we tested the enzymatic activity of the recombinant enzyme. The only products found by radio-HPLC analysis were (9*Z*)-12-oxo-9-dodecenoic acid and 9-oxo nonanoic acid, respectively, the expected reaction products of a HPL enzyme. Ketols, products of AOS activity, or divinyl ethers resulting from DES activity were not observed (Fig. 2). We conclude from these results that the isolated cDNA encodes for an HPL. In order to characterize the range of accepted substrates for the novel HPL enzyme, different hydroperoxides of linoleic and α-linolenic acid were tested as substrates and the

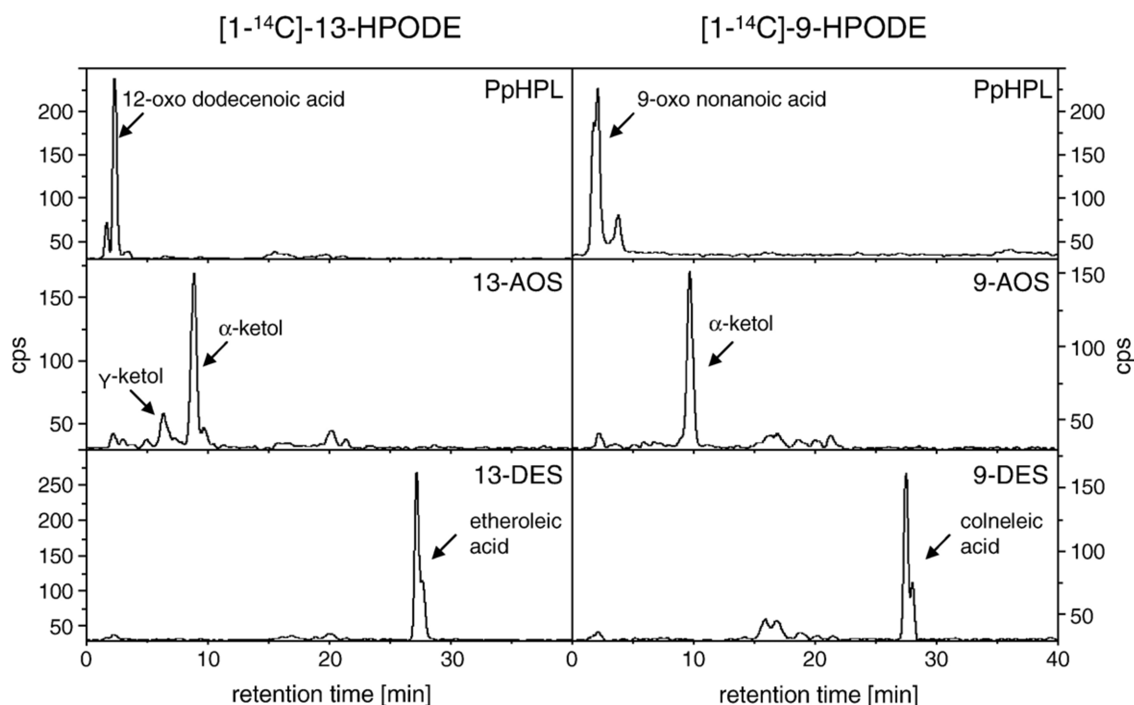

Fig. 2. Product analysis of PpHPL. Radiochromatograms of products formed after incubation of either [1-^{14}C]-13-HPODE or [1-^{14}C]-9-HPODE with recombinant enzymes as indicated.

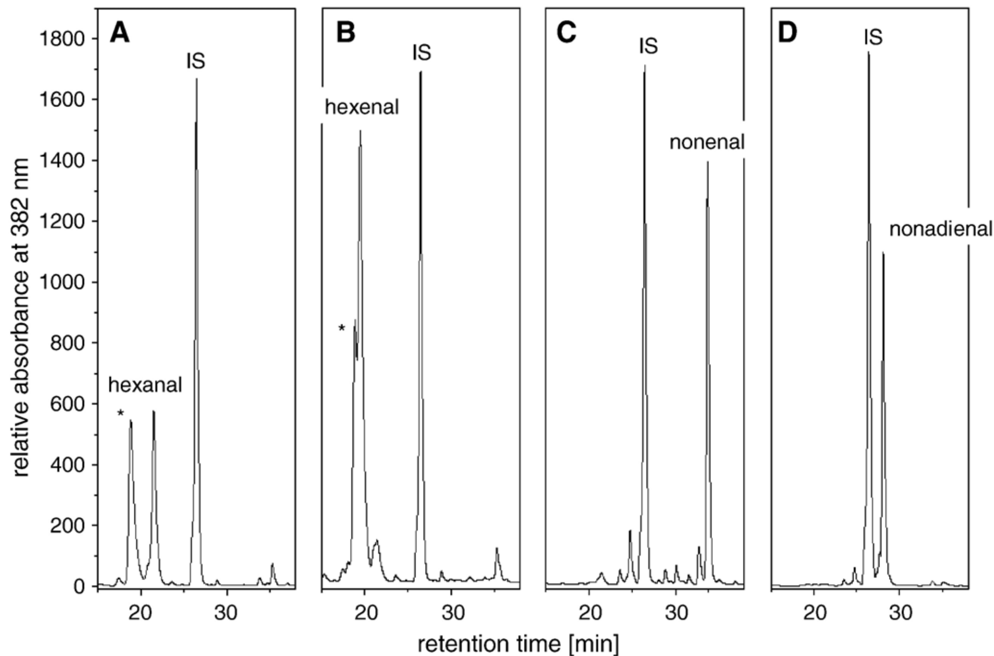

Fig. 3. Analysis of formed aldehydes. HPLC-analysis after incubation of recombinant PpHPL with 13-HPODE (A), 13-HPOTE (B), 9-HPODE (C) and 9-HPOTE (D). Samples were derivatized with DNPH before analysis. IS = internal standard (heptanal). * = 12-oxo-9-dodecenoic acid.

resulting aldehydes were detected as DNPH-derivatives in HPLC-analyses (Fig. 3). All tested fatty acid hydroperoxides of linoleic and α-linolenic acid were metabolized by the enzyme indicating that this HPL may belong to the group of unspecific 9/13-HPLs. Using 9-HPODE as substrate, we determined the pH optimum as well as kinetic parameters of the recombinant protein spectrophotometrically. PpHPL exhibited a pH optimum of about pH 8.0. Assuming the total protein content of purified recombinant PpHPL to be active, the specific activity was 532.3 U/mg and the K_m was 61.7 μM.

To gain further insight into the substrate specificity of the enzyme in addition to the previous substrates, the hydroperoxides of γ-linolenic and arachidonic acid were tested. The majority of hydroperoxides tested served as substrates and were

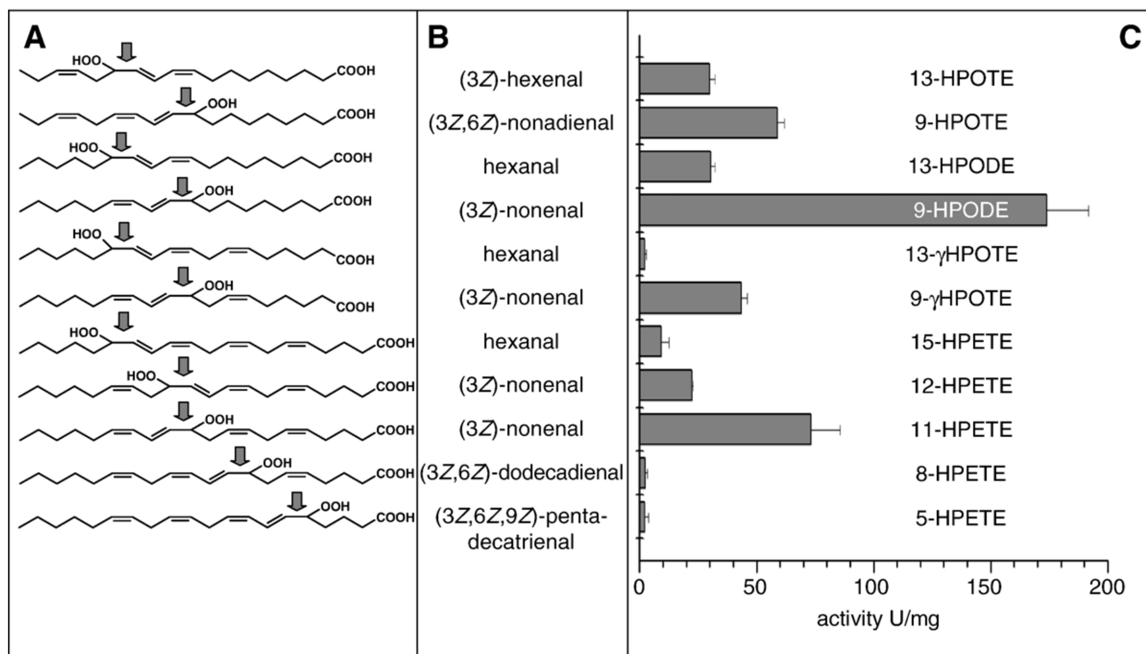

Fig. 4. Substrate specificity of recombinant PpHPL. (A) Structures of hydroperoxide substrates administered, the arrows indicate cleavage site by PpHPL; (B) Aldehydes formed after incubation with correspondent hydroperoxide. Aldehydes were identified as DNPH-derivatives by HPLC-DAD or HPLC-MS, respectively; (C) Relative activity of PpHPL against hydroperoxides; recombinant protein was incubated with hydroperoxide in 100 mM sodium phosphate, pH 8.0, and activity was measured spectrophotometrically at 234 nm. Error bars are based on S.D. ($n = 3$).

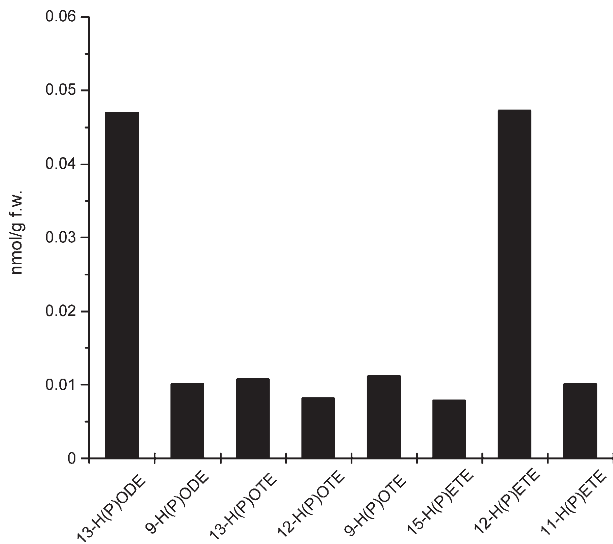

Fig. 5. Fatty acid hydro(pero)xides of wild type moss. The amount of hydro(pero)xides was determined in protonema of wild type moss as described under Materials and methods. The figure shows mean values of two independent experiments.

converted to the corresponding aldehydes (Fig. 4). Fig. 4B shows the detected products with aldehyde functionality. In each case we found an additional aldehyde compared to the negative empty vector control. The identity of derivatized aldehydes was verified in HPLC runs by coelution with authentic standards. The aldehydes derived from 8- and 5-HPETE were separated on HPLC and analyzed directly by electrospray ionization/mass spectrometry. Their molecular weight and retention time matched those of C12- and C15-aldehydes. Based on biosynthetic considerations their structures were tentatively assigned to (3*Z*,6*Z*)-dodecadienal and (3*Z*,6*Z*,9*Z*)-pentadecatrienal. To analyze the substrate specificity on quantitative basis we used 30 μM of each hydroperoxide in a spectrophotometric assay where the consumption of the hydroperoxide was followed. The highest activity was observed using 9-HPODE as substrate, followed by 11-HPETE (40%), 9-HPOTE (35%), and 9-γHPOTE (26%). Using 13-γHPOTE, 8-HPETE and 5-HPETE the activity was not easily detectable (below 1% in comparison to 9-HPODE, Fig. 4C). Nevertheless the resulting aldehydes were reproducibly detected by HPLC analysis after 30 min of incubation time of the enzymatic reaction, whereas the negative empty vector control failed the produce such aldehydes.

3.2. Determination of fatty acid hydro(pero)xides

Fatty acid hydroperoxides are the direct precursors of volatile oxylipins. Since they are reactive substances which are not fully stable under our work up procedure their amount was expressed as a mixture out of hydroperoxy and hydroxy fatty acid derivatives. The amount of hydro(pero)xides was analyzed in protonema of wild type moss to get first hints for the possible endogenous substrates of *CYP74* enzymes. The predominant fatty acid hydro(pero)xides were 13-H(P)ODE and 12-H(P)ETE both accumulating to about 0.047 nmol/g fresh weight (Fig. 5). Other detected hydroperoxides were only found at a basal level of about 0.01 nmol/g fresh weight.

3.3. Intracellular localization

The enzymatic activity of *CYP74*-enzymes was previously found to associate with plastids, in particular with their envelope [30]. This observation is consistent with the prediction that some of the *CYP74*-enzymes carry a plastidic transit peptide. Import assays as well as immunocytochemical studies with different *CYP74*-enzymes support their plastidic localization [12,13]. We created an expression vector encoding a C-terminal fusion protein of PpHPL with YFP and transformed *Physcomitrella* protoplasts with this construct. After 3 days of expression

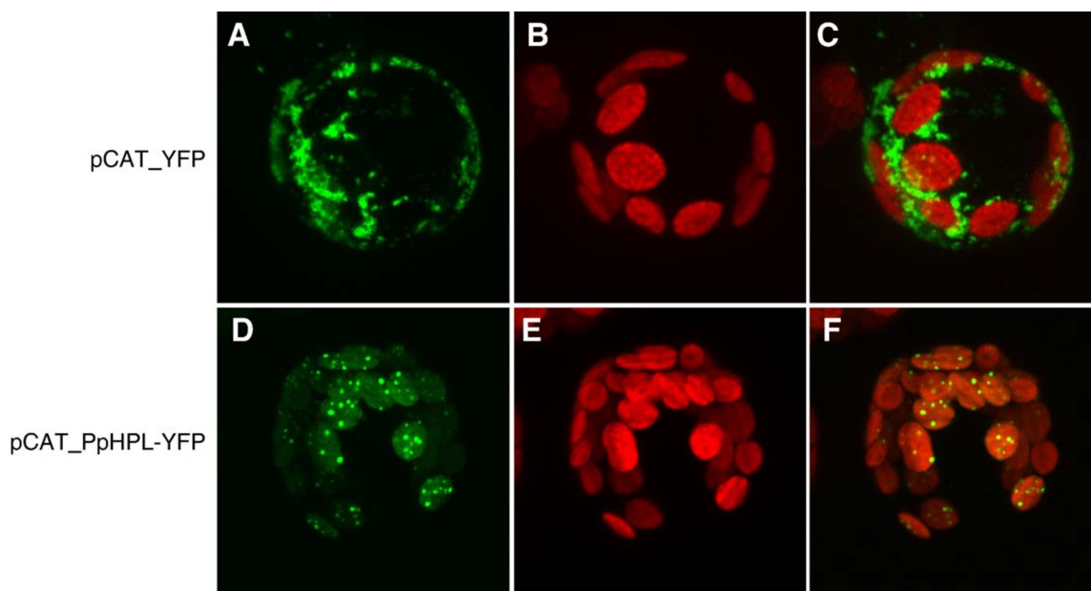

Fig. 6. Subcellular localization of PpHPL. *P. patens* protoplasts were transfected with pCAT_YFP and pCAT_PpHPL-YFP; (A, D) YFP fluorescence in a transfected protoplast. (B, E) Chlorophyll fluorescence of the same protoplast. (C, F) Merged image of A and B or D and C, respectively.

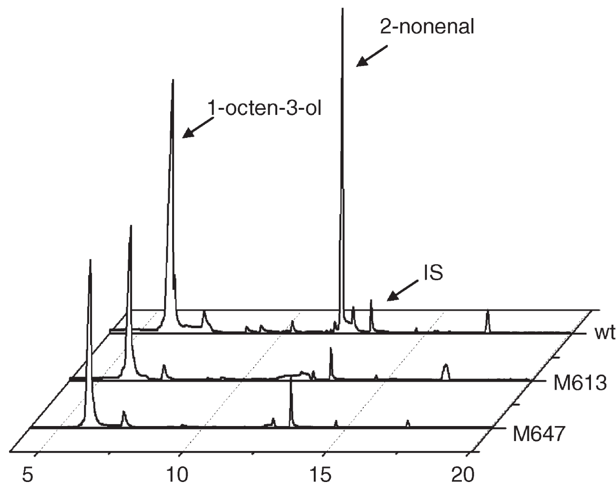

Fig. 7. SPME-analysis (GC/MS total ion chromatogram) of *P. patens* wild type and PpHPL-KOs volatile formation after wounding. Wild type (wt) and two knock-out lines (M613, M647) were wounded for 2 min. I.S., internal standard (2-decanon). Arrows indicate the emitted major volatiles.

YFP fluorescence was detectable. Visualization of the YFP fusion protein by fluorescence microscopy revealed a plastidic localization. To analyze it in more detail, we used confocal laser scanning microscopy. As seen in Fig. 6A YFP protein not fused to an HPL enzyme is distributed within the cytosol. In the case of PpHPL-YFP plastidic localization was obvious by colocalization with red chlorophyll autofluorescence. Interestingly, the distribution of the fusion protein in chloroplasts was not uniform, and a spotted pattern of localization was observed (Fig. 6D,F).

3.4. Analysis of PpHPL knock-out mosses

Recently it was described that after addition of arachidonic acid to wounded moss different volatiles like oct-1-en-3-ol, oct-3-en-1-ol and (2*E*)-nonenal were emitted [25]. The production of the octenols was caused by the recently cloned LOX of *P. patens* [26]. To analyze if the HPL cloned here is responsible for the formation of (3*Z*)-nonenal, which could be converted to (2*E*)-nonenal by an isomerase, we created knock-out mosses where the *PpHPL*-gene was disrupted by a cDNA coding for kanamycin resistance. We obtained 30 independent transgenic lines and the integration into the *PpHPL*-gene was verified by PCR as described in experimental procedures. Four independent lines were chosen for further studies. The knock-out lines showed normal development and analysis on minimal medium showed no visible phenotype. Since no pathogens for the moss are yet available, it was not possible to test whether these mutant lines were impaired in pathogen defense. However, all were

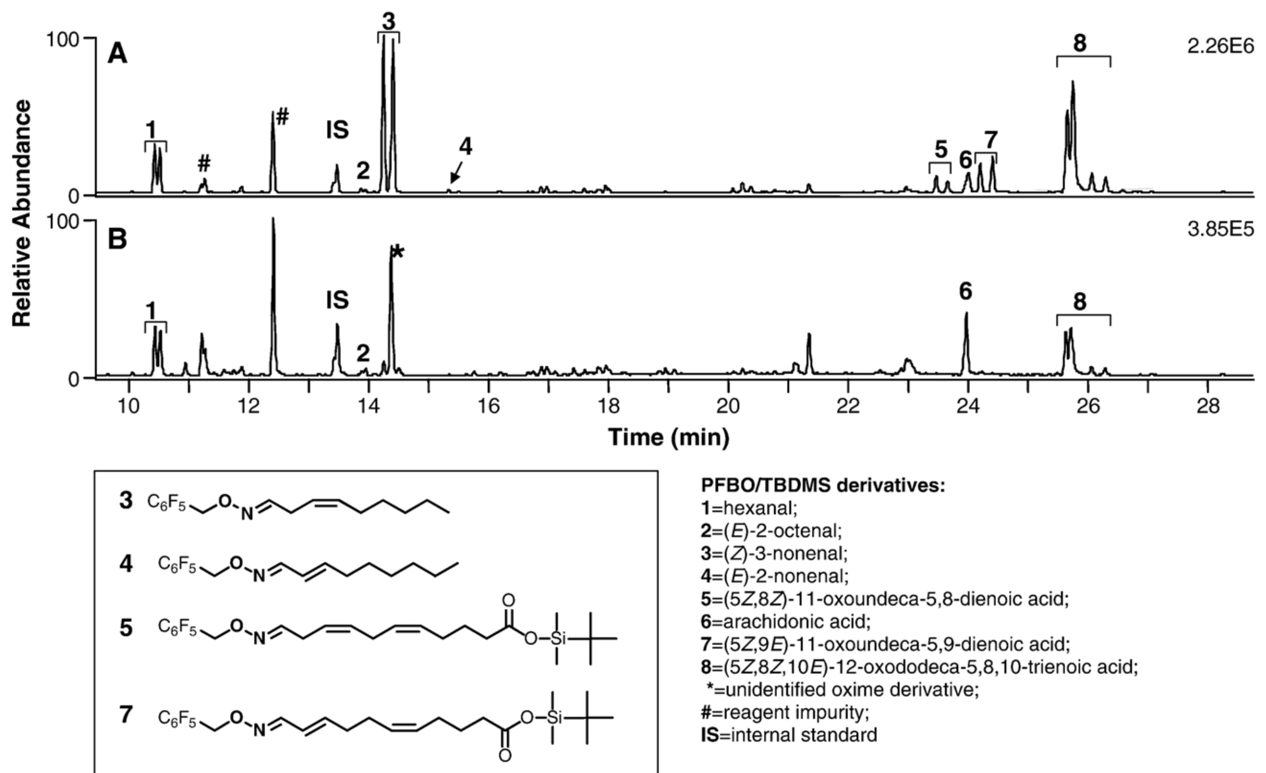

Fig. 8. In situ trapping of oxylipins harboring an aldehyde functionality after administration of arachidonic acid and pentafluorobenzyloxime to the medium of wounded WT (A) and PpHPL knock-out mutant (B). The chromatograms show the ion trace of the key ion of *m*/*z* 181. The reaction was stopped after 30 min and the extract was silylated with MTBSTA [40]. The picture shows the gas chromatographic separation of the derivates. While the WT produces elevated amounts of (3*Z*)-nonenal (3) and the 11-oxo fatty acid (5,7), the PpHPL knock-out mutant lacks these compounds. The 12-oxo fatty acid (8) is still found in elevated amounts in that knock-out.

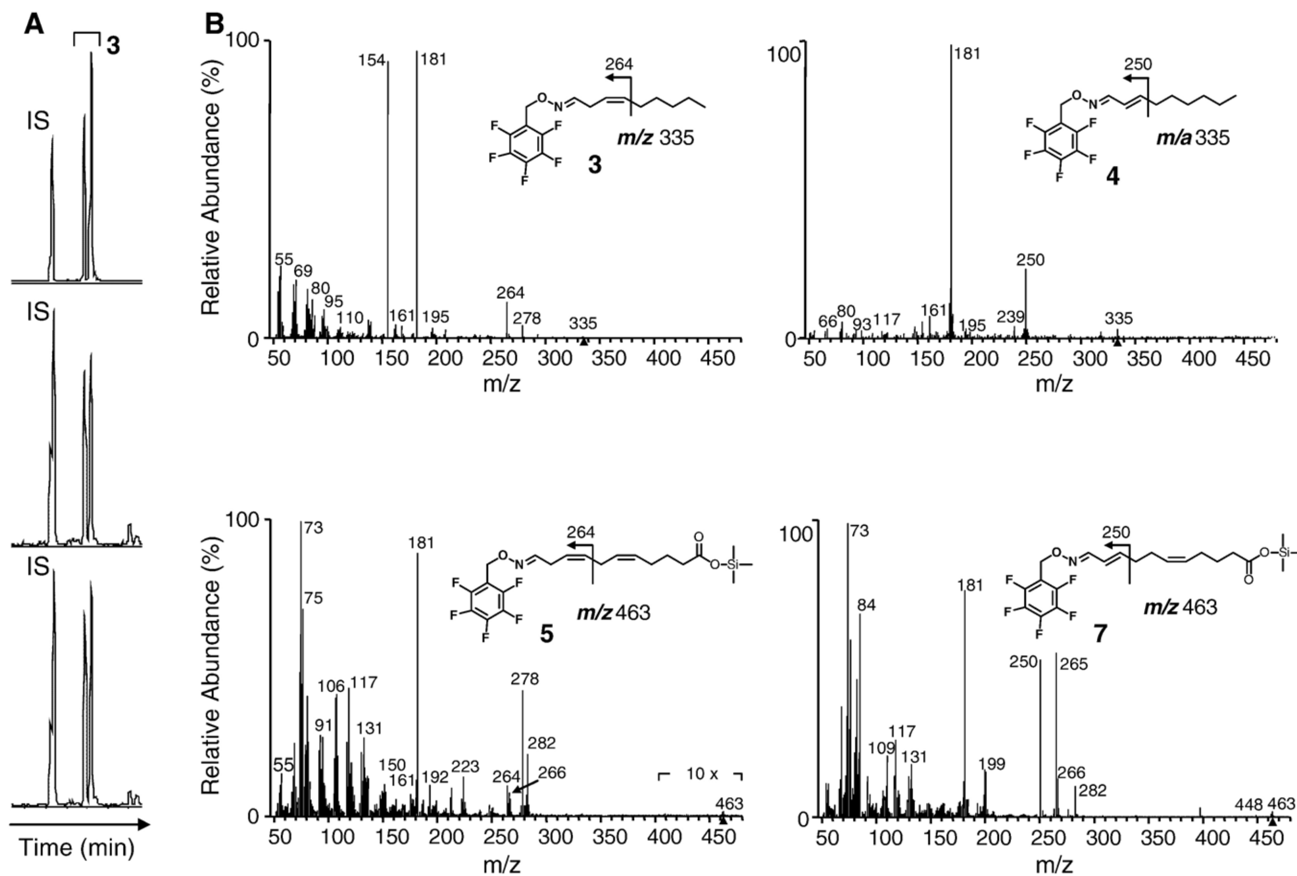

Fig. 9. Identification and structure elucidation of (3*Z*)-nonenal and 11-oxo fatty acids. (A) Co-injection of the derivatized standard of (3*Z*)-nonenal. The chromatograms show the ion trace of *m/z* 181 (IS = oxime derivative of benzaldehyde) 3. From top to bottom: Synthetic reference; extracted sample; co-injection at a ratio of 1:4. (B) Mass spectra of the derivatives of the nonenals 3 and 4 and the oxo acids 5 and 7. The structure of 4 has been previously documented by co-injection with commercial available standard [25]. Characteristic fragments of 264 (5) and 250 (7) allow the assignment of the double bond positions in the oxo acids (see Results).

deficient for HPL activity. Thus the formation of volatile oxylipins after exogenous addition of arachidonic acid was compared. As described for the wild type [25], we observed within the first 30 s the transient formation of (12*S*)-H(P)ETE and only trace amounts of racemic 11- and 15-H(P)ETE. The amount of (12*S*)-H(P)ETE declined then within the next 15 min and formation of oct-1-en-3-ol, oct-3-en-1-ol and (2*E*)-nonenal was observed. Addition of neither linoleic nor linolenic acid led to formation of volatiles under these conditions. The release of volatiles from moss lines sonicated for 2 min in an ice bath were analyzed by headspace analysis (Fig. 7). In the wild type moss we observed the production of octenols and (2*E*)-nonenal. In contrast no (2*E*)-nonenal was detected in two knock-out moss lines analyzed. The production of other volatiles was not affected in these knock-out lines. Interestingly, hexanal production was still observed in the knock out lines, even if the HPL is able to form this aldehyde from 13-HPODE in vitro (Fig. 8). The ω-oxo fatty acid (11-oxo undecadienoic acid), the other product of the HPL reaction, was also not detectable in the knock out mutants, whereas 12-oxo dodecantrienoic acid, the product of the reaction with the multifunctional LOX, was found (Fig. 8).

With pentafluorobenzyloxime trapping experiments, we could show that the wild type moss initially releases (3*Z*)-nonenal (Fig. 8), a product also formed with the recombinant enzyme. In contrast, investigations of the gas phase over the wounded moss with solid phase microextraction indicated the presence of (2*E*)-nonenal and no traces of the (3*Z*)-isomer were found. This indicates that an additional isomerizing activity existed in the crude moss preparations. Structures of (2*E*)- and (3*Z*)-nonenal were proven by co-injection of a commercial available or synthetic standard, respectively (Fig. 9A) [40]. The unsaturated ω-oxo fatty acids were initially detected as *tert*-butyldimetylsilyl-oxime-derivatives (Fig. 8), where the molecular mass can be easily determined by the detectable molecular ion and the M-57 fragment [40]. For further structure elucidation the carboxylic group was silylated with MSTFA and the aldehyde derivatized by pentafluorobenzyloxime treatment. The molecular ions of trimethylsilyl derivatives of the 11-oxo fatty acids are of low intensity. Nevertheless the molecular mass can be deduced from peaks which correspond to a loss of 181 u (*m/z* 282) and 197 u (*m/z* 266) (Fig. 9). The mass spectra allowed moreover the assignment of the double bond position in comparison with literature data of similar compounds [41]. The position of the α,β-double bond of the 11-oxo fatty acid 7 can be deduced from the diagnostic ion of mass 250 [42], a fragment also found in (2*E*)-nonenal 4 (Fig. 9). In case of the 11-

oxo fatty acid 5, where the double bonds are not conjugated, this key ion is not dominant, the same is true for the fragmentation pattern of (3*Z*)-nonenal 3 (Fig. 9). For these latter metabolites fragments with the masses of 264 are abundant, which are indicative for an α-fragmentation adjacent to the isolated double bond (Fig. 9). Presence of this fragment in the mass spectrum of the 11-oxo fatty acid 5 also excludes the presence of a conjugated double bond system. Together with biosynthetic considerations a (5*Z*,8*Z*)-double bond configuration of 5 is highly likely. Methylation instead of silylation of the carboxylic group supported this interpretation further, all key fragments according to [41] for α,β-unsaturated aldehydes could be detected.

4. Discussion

In contrast to flowering plants *P. patens* harbors high proportions of arachidonic acid (up to 30% of total fatty acids) besides C18 PUFAs [43]. *P. patens* exploits arachidonic acid for the formation of its major oxylipin-volatiles. We investigated details on how arachidonic acid, which is first oxidized to 12-HPETE [25], is further metabolized to a variety of different shorter chain oxylipins. 12-HPETE may be cleaved either by a typical plant-type HPL, belonging to *CYP74* enzymes (this report), leading to the formation of (3*Z*)-nonenal. Further fast isomerization of (3*Z*)-nonenal to the (2*E*)-isomer occurs by a separate isomerizing activity. An alternative source for the moss-volatiles is an unusual lyase activity of a LOX producing not only hydroperoxy fatty acids but also octenal and octenol isomers [26]. We describe here the isolation and characterization of the first HPL from a lower plant belonging like its homologues from flowering plants to the *CYP74* subfamily of P450 enzymes. By homology searches in EST-databases containing about 140,000 ESTs from *P. patens*, we found two ESTs with homology to *CYP74*-enzymes from flowering plants. One EST was completed by screening a phage library and the full length coding sequence was expressed in *E. coli*. The recombinant protein exhibited no activity, but an amino terminally truncated protein cleaved hydroperoxides into aldehydes (Fig. 3) and ω-oxo fatty acids. This property classified it as HPL belonging to *CYP74*-enzyme family [14]. The amino terminal region was identified as plastidic transit peptide using the prediction program TargetP. Several other *CYP74*-enzymes contain also such a transit peptide and show only as truncated versions enzymatic activity [11,44–46]. On amino acid sequence level, PpHPL shows a similarity of about 61% and identity of about 42% to an AOS from *Arabidopsis*, belonging to the *CYP74A* subfamily [11]. But in comparison to the other subfamilies (*CYP74B*-*CYP74D*), the values are nearly the same: CaHPL (*CYP74B*) 57% similarity and 40% identity, MtHPL (*CYP74C*) 59% similarity and 39% identity and NtDES (*CYP74D*) 57% similarity and 40% identity. These low and more or less equal levels may be due to the high phylogenetic distance between the moss and the flowering plants [47], thus making it difficult to decide to which subfamily PpHPL should belong to or whether it should belong to a new subfamily *CYP74E*.

Most HPLs cloned to date belong to the *CYP74B* subfamily. The substrate specificity of some recombinant enzymes of this subfamily has been investigated and found to have a high selectivity for 13-HPOTE [44–46]. Their activity against other hydroperoxides was very low (13-HPODE 7–15% and 9-hydroperoxides 0–9%). Similar results were observed when purified HPLs from plant tissues like green bell pepper fruits or tea leaves were analyzed [48,49]. In these examples unusual plant hydroperoxides like 9- or 13-γHPOTE were only poorly converted (0–2% relative to 13-HPOTE). Thus, it was proposed that the common feature of the substrates seems to be an ω6-hydroperoxide adjacent to a triene motif. Changes in *Z*–*E*-geometry [49] as well as elimination of the ω3-double bond lead to a dramatic decrease of the conversion rate [48]. The HPL from *Arabidopsis* is an exception, here 9-γHPOTE is converted with about 50% efficiency in comparison to the best substrate 13-HPOTE [50]. An explanation might be that both substrates contain the 4-hydroperoxy-(1*Z*,5*E*,7*Z*)-diene structure and only the orientation with respect to the carboxyl group differs. The influence of the length of the carbon chain was investigated using tea leaf HPL [48]. Here, hydroperoxides with carbon chain length ranging from C14 to C24 containing the ω6-hydroperoxide-triene motif were analyzed. Interestingly, the highest activity was detected using the C22-hydroperoxide, which is not present as a substrate in plants. In contrast to these 13-hydroperoxide-specific enzymes (13-HPLs), there were also HPLs having a broad substrate spectrum (unspecific or 9/13-HPLs). While the 13-HPLs belong to the *CYP74B* subfamily, unspecific HPLs are members of the *CYP74C* subfamily and have got the highest sequence similarity to AOS enzymes [4]. The 9/13-HPL from cucumber shows the highest activity towards 9-HPODE [51], whereas the HPL from melon prefers 9-HPOTE [52]. The activity against the other hydroperoxides ranged from 80% to 22%. Because of limited information, it is quite difficult to find conserved substrate motifs for this type of enzymes. The PpHPL shows the highest sequence similarity also to AOS enzymes and has also a slight preference to 9-HPODE but its activity against other hydroperoxides ranges from 5 to 40% (11-HPETE) (Fig. 4C). Thus we prefer to classify the enzyme as unspecific 9/13-HPL. In contrast to all other enzymes analyzed so far, the four best substrates of PpHPL shared an ω10-hydroperoxide-diene motif, whereas additional double bonds like in 9-HPOTE or 9-γHPOTE reduce drastically its activity.

Taken together, essential for optimal activity of HPLs is a *cis*–*trans*-configurated conjugated diene system with the *trans*-double bond beside the (*S*)-hydroperoxide bearing carbon atom. This system is always created by LOXs after metabolizing a (1*Z*,4*Z*)-diene system. Additional double bonds influence the activity rate: In case of 13-HPLs, a double bond at the other site of the hydroperoxide group leads to an increase in activity [6,14] whereas in case of cucumber HPL [51] and PpHPL the activity decreased. Analyses of the specificity of the tea leaf HPL, a 13-HPL, and our results indicated that the absolute position of the diene system in relation to the methylene end and not to the carboxyl end is an important factor for the substrate specificity. Nevertheless the carboxy group plays also a role, since methylation reduces often the activity whereas to some extent an acid-amine is a better substrate [50,53]. An influence of the pH is discussed in respect to amount of carboxylate anion in relation to the undissociated carboxyl group [54].

The enzymatic activity of *CYP74*-enzymes was often found associated with the plastid. Import assay studies with tomato AOS and HPL showed also an association with this organelle [12]. PpHPL also apparently contains a plastidic transit peptide and the PpHPL-YFP fusion protein was guided to the chloroplast (Fig. 6D,F). Since the enzyme is only active without its transit peptide we assume that PpHPL is imported into the chloroplast where a transit peptidase processes the protein. The pH optimum of PpHPL (around pH 8.0) supports also a plastidic localization. At neutral pH, there is still 80% of the activity suggesting that PpHPL could be active during photosynthetic and non-photosynthetic periods. During the purification of recombinant PpHPL, we observed an association of the protein with the *E. coli* membranes as it is described also for other *CYP74*s [55,56]. Because of that and the non-uniform distribution of PpHPL-YFP fusion protein in the chloroplast, we assume that the protein is somehow linked to special lipid domains within membranes that might be the inner envelope or the thylakoid.

It was recently described that after wounding the moss emitted a specific blend of volatiles that yet has not been found in flowering plants [25]. As main constituents we identified oct-1-en-3-ol, oct-3-en-1-ol and (2*E*)-nonenal, which were exclusively derived from arachidonic acid. While the production of the octenols is caused by the recently cloned LOX of *P. patens* [26], the HPL described here is responsible for the formation of (3*Z*)-nonenal as could be shown by knock out experiments. The analysis of two independent knock out lines show that PpHPL is only involved in the generation of (3*Z*)-nonenal which is rapidly transformed by an isomerase activity to the (2*E*)-isomer in the wild type (Fig. 7). The formation of octenols and the formation of hexanal is still observed in the knock out lines (Fig. 8). In this context it is interesting to note that the recombinant HPL is capable of transforming the precursor of hexanal 13-HPODE in vitro (Figs. 3 and 4), a hydroperoxide found in elevated amounts in the moss (Fig. 5). Apparently, other resources are as well responsible for the formation of this shorter chain length aldehyde. The specific transformation of 12-HPETE by PpHPL, while 13-HPODE seems not to be accessible for the enzyme cannot be easily explained by different locations of both substrates. Analysis of location of precursor fatty acids linoleic and arachidonic acid revealed an equal distribution of both fatty acids in all lipid classes detected in the moss and these data supported earlier findings on analysis of fatty acids in crude lipid fractions of this moss [57]. Therefore it will be interesting to identify the additional hexanal forming activity in the future. It is likely to be different from that of flowering plants since the available EST collection lacks a readily identifiable additional sequence coding for another HPL.

According to our findings described here and in recent publications [25,26], a new picture for the wound activated formation of volatile oxylipins in *P. patens* arises that involves only one key enzyme required for the activation of arachidonic acid. This single LOX (PpLOX1) can account itself for high product diversity [26]. It either produces directly octenols from arachidonic acid or it provides the substrate (12-HPETE) for PpHPL described here, leading to formation of (3*Z*)-nonenal, which is subsequently isomerized. Although 12-HPETE is not the best substrate of the recombinant enzyme, incubation of the moss with different fatty acids showed the increased formation of volatiles only in case of arachidonic acid via the production of 12-HPETE [25]. Analysis of the content fatty acid hydro (pero)xides shows a high level of 13-H(P)ODE and 12-H(P) ETE. The resulting HPL-products may be hexanal and (3*Z*)-nonenal. Interestingly only one of these aldehydes (3*Z*)-nonenal is reduced in the PpHPL knock out lines, which indicates that only 12-HPETE may be the endogenous substrate of PpHPL. So the specific formation of 12-HPETE determines the specificity of the HPL reaction although the recombinant PpHPL has a substrate preference for 9-HPODE. Thus, the moss produces metabolites typical for animals, plants, algae, and mushrooms by new transformations of arachidonic acid, combining in an unique way metabolic themes from all these organisms. This might be the reason why mosses are known to be highly resistant to herbivores and pathogens in contrast to less resistant flowering plants where this type of resistance is often mediated only by a single LOX/HPL pathway. It will be interesting to test this hypothesis in the future as soon as bioassays testing the activation of defense-response become available for *P. patens*.

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Formation of Halogenated Medium Chain Hydrocarbons by a Lipoxygenase/ Hydroperoxide Halolyase-Mediated Transformation in Planktonic Microalgae

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Marine organisms are a rich source for halogenated metabolites. These range from simple halomethanes produced in macroalgae to the most complex secondary metabolites found in sponges, ascidia, and other invertebrates.[1,2] Nevertheless, only few pathways for enzymatic halogenation are known.[2] Among the halogenating enzymes, haloperoxidases are the most widely distributed, although $FADH_2$-dependent halogenases and *S*-adenosylmethionine transforming fluorinating enzymes have been identified, as well.[3] Recently, a novel class of non-heme Fe^{2+}, α-ketoglutarate, and O_2-dependent halogenases has been added to this known enzymatic repertoire.[4] Several halogenated fatty acids and fatty acid derived metabolites have been previously reported. In most cases, their biosynthesis is dependent on the above-mentioned pathways, but few products, for which the biosynthesis might be explained by nucleophilic attack of a halogenide, have been identified, as well.[5] Here we describe a new pathway to halogenated natural products in the marine diatom *Stephanopyxis turris*.

Diatoms are very abundant unicellular algae at the bottom of the marine food chain. Their primary production is responsible for more than 20% of the global carbon fixation. In recent years, certain diatom species came into the focus of chemical ecologists due to the production of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes, such as 2,4-octadienal and 2,4,7-decatrienal. This aldehyde release is suggested to be involved in an indirect chemical defense targeted against herbivores.[6] The volatile unsaturated aldehydes, as well as ω-oxo acids, such as (5*Z*,8*Z*,10*E*)-12-oxo-5,8,10-dodecatrienoic acid (12-ODTE **9**), are produced via lipoxygenase-mediated pathways which are initiated upon cell disruption.[6,7] During a screening of diatoms for their ability to generate unsaturated aldehydes, we revealed that more than 30% of the investigated species produce these potential defensive metabolites.[8] Included is *S. turris*, which releases 12-ODTE **9**, a metabolite earlier described from the freshwater diatom *Asterionella formosa*.[7] In this alga, eicosapentaenoic acid **1** is transformed to 12-ODTE **9** and 1,3*E*,5*Z*-octatriene.[7] Contrary to our expectations, *S. turris* did not produce elevated amounts of the octatriene, indicating that another pathway is involved in the formation of **9**. Since the wounded algae exhibited an intense flowery smell, we concluded that other volatiles are most likely formed during fatty acid transformation. For characterization of this bouquet, we performed a headspace solid-phase microextraction (SPME)[7] of stationary cultures (10−40 mL, 1×10^4 cells mL^{-1}), which had been concentrated by filtration prior to wounding by sonication.[8] One dominant volatile metabolite and several minor components were identified (Figure 1). The characteristic isotope patterns of the molecular ions pointed to chlorinated volatiles. EI spectra showed a dominant loss of M-35, and the accurate mass was in agreement with chlorinated unsaturated C8 hydrocarbons (TOF-MS $^{12}C_8{}^1H_{13}{}^{35}Cl$ calculated, 144.0706; measured, 144.0718 (**3**), 144.0706 (**4**)). Characteristic fragments (e.g., base peak m/z = 69) suggested that an unsaturated fatty acid derived C5 terminus is still present (Figure 2). Following biosynthetic considerations, we proposed the structures of (5*Z*)-3-chloro-1,5-octadiene **3**, (2*Z*,5*Z*)-1-chloro-2,5-octadiene **4**, and its (2*E*,5*Z*)-isomer **5**. Retention time and mass spectra of synthetic standards matched those of the algal metabolites confirming their identity.

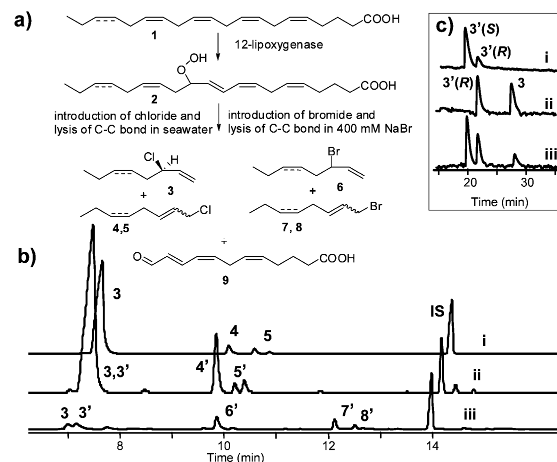

Figure 1. (a) Lipoxygenase/hydroperoxide halolyase-mediated transformation of eicosanoids. The numbers refer to metabolites derived from eicosapentaenoic acid **1** (dashed double bond present); numbers with a ′ refer to ω6-arachidonic acid derived metabolites (dashed double bond not present). (b) (i) SPME of *S. turris* wounded in seawater; (ii) as (i) in the presence of ω6-[$^2H_8$]-arachidonic acid; and (iii) in the presence of ω6-arachidonic acid in 400 mM buffered NaBr, IS = 2-decanone. (c) The inset shows the chiral GC separation of **3** and **3′**: (i) synthetic mixture of predominantly (3*S*)-**3′**; (ii) *S. turris* extract; (iii) co-injection.

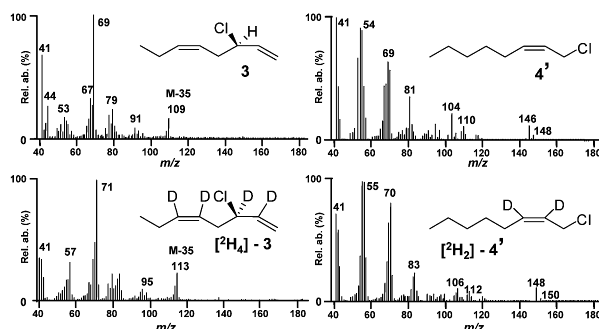

Figure 2. Top: MS (EI) spectra of *S. turris* volatiles. Below: Transformation products of ω3-[$^2H_8$]-arachidonic acid (left) and ω6-[$^2H_8$]-arachidonic acid (right).

The simultaneous production of C12 and C8 metabolites suggested that *S. turris* transforms C20 fatty acids by a new halogenating lyase activity. For further elucidation of the mechanism, ω6-[$^2H_8$]-labeled arachidonic acid **1′** was applied to *S. turris*. The mass shift of Δ6 observed in LC/MS experiments indicated that the

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labeled precursor was incorporated (rate ca. 27%) into 12-ODTE **9**. In parallel, we could identify labeled 1- and 3-chlorooctenes **3′**−**5′** (Figures 1 and 2). If ω3-[$^2H_8$]-labeled arachidonic acid was administered, the chlorooctadienes **3**−**5** were formed, but (8*Z*,10*E*)-12-oxo-8,10-dodecadienoic acid was found as the second fragment (Supporting Information). These experiments show that C20 fatty acids with varying degree of unsaturation and varying double bond position are transformed into C12-oxo acids and C8 halocarbons by *S. turris*.

At this stage, it was not clear if the halocarbons are formed directly by an enzyme-controlled reaction or if an abiotic transformation in the NaCl-rich seawater is responsible for the halogenation. Since such a secondary reaction would result in racemization, the stereochemistry of enzymatically produced 3-chlorooct-1-ene **3′** was investigated. Chiral GC/FID analysis of the enzyme product was carried out in comparison with a synthetic standard (Supporting Information). 3-Chlorooct-1-ene **3′** is of >98% ee (3*R*) (Figure 1) and thus directly generated by enzymatic halogenation. Since no racemization occurred and since the product ratio of **3** to **4** and **5** remained constant even after prolonged exposure to seawater, we conclude that the involved enzyme(s) release a mixture of these chlorinated C8 metabolites.

Incubations of *S. turris* in different media revealed that the introduced chlorine originates from seawater. If a cell pellet was resuspended in deionized water before sonication, no formation of halogenated metabolites and 12-ODTE **9** was observed (data not shown). However, if the algae were incubated in 100 mM Tris/HCl pH 7.8 containing 400 mM NaBr, the preferred formation of 1- and 3-bromooctadienols (**6**−**8**) was observed (Figure 1, Supporting Information). The supply of halogen ions from the surrounding water evidently determines product formation. The pseudohalogene SCN^- was not accepted, and NaF inhibited product formation completely (see below).

For the further characterization of the transformation resulting in the cleavage of a hydrocarbon chain and the introduction of a halogen, prototypic reactions might not be adopted. However, it has been observed in the moss *Physcomitrella patens* that arachidonic acid can be activated to 12-hydroperoxyeicosatetraenoic acid (12-HpETE), which is further transformed to 12-ODTE **9** and a mixture of 1- and 3*R*-octenol.[9] Both, the introduction of oxygen and the subsequent cleavage of the intermediate are catalyzed by a multifunctional lipoxygenase.[9] Similarity of the 12-ODTE **9** precursor and the kinetics of oxo acid formation (Supporting Information) prompted the investigation for related fatty acid activation in *S. turris*. On addition of the hydroperoxide reducing agent ebselen[10] (100 μM final concentration) and 100 μg of ω6-[$^2H_8$]-arachidonic acid before cell disruption, the intermediate [$^2H_8$]-12-HpETE could be trapped as the corresponding hydroxy fatty acid and characterized in LC/MS experiments (Supporting Information). This experiment confirms a pronounced lipoxygenase activity in the wounded cells. Incubation of *S. turris* with 2 μg of synthetic 12-HpETE proved that this hydroperoxide is indeed a substrate for the halogenating enzyme. In the absence of this substrate, *S. turris* only releases the chlorooctadienes **3**−**5** from eicosapentaenoic acid since it lacks endogenous arachidonic acid for the production of the chlorooctenes **3′**−**5′** (verified by analysis of the total fatty acids). After addition of 12-HpETE, however, de novo production of **3′**−**5′** from this intermediate was observed. As a control, the cell pellet was heated for 15 min to 120 °C before adding 12-HpETE. This treatment completely inactivated the involved enzymes and prevented the reaction. Hydroperoxy fatty acids are thus direct intermediates en route to the chlorinated products. Addition of 200 mM NaF afforded decoupling of the lipoxygenase and the halogenating activity. NaF exclusively inhibits fatty acid hydroperoxide formation due to the formation of a fluoride−Fe_3^+ complex,[11] leaving the transformation of externally supplied 12-HpETE to 12-ODTE and **3′**−**5′** active (Supporting Information). On the basis of our findings, we propose the name hydroperoxide halolyase for the newly characterized enzyme. However, the detailed enzyme mechanism has yet to be fully established.

In summary, we have shown that *S. turris* transforms C20 fatty acids by a lipoxygenase-mediated reaction to intermediate hydroperoxides. These are then employed for the production of new halogenated metabolites by an unprecedented pathway (Figure 1). Our work thus adds a fundamentally new biogenetic transformation to the limited set of known halogenating enzymatic reactions. It is especially interesting in the context of biogeochemical cycling to identify a planktonic alga as a new source for halocarbons. Halogenated metabolites play a significant role for the functioning of atmospheric processes.[12] Until now, mainly methyl halides from microalgae have been considered from plankton.[13] Adding a new and potentially widely distributed marine source of halogenated metabolites might influence the general view of plankton impact on atmospheric processes. Further studies will have to show if the newly identified metabolites contribute to infochemical-mediated processes of microalgae.

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Supporting Information Available: Additional figures showing the decoupling of lipoxygenase and the tentative hydroperoxide halolyase activity, trapping of 12-HpETE and the time course of 12-ODTE formation, as well as experimental details. This material is available free charged via the Internet at http://pubs.acs.org.

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Influence of diatoms on copepod reproduction. I. Field and laboratory observations related to *Calanus helgolandicus* egg production

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ABSTRACT: Egg production rates (EPR) by *Calanus helgolandicus* females were investigated with specimens sampled weekly, from April to November 2003 and from March to October 2004, at a station located in the English Channel off Roscoff. Comparison of results between 1994, 2003 and 2004 showed that *C. helgolandicus* was a late spawner in 1994 and became an early spawner in 2003 and 2004. In all cases high variations in EPR were observed, which could not be correlated to phytoplankton biomass, expressed as diatom, chlorophyll *a*, particulate carbon and nitrogen concentrations in 2003 and 2004. Neither were they correlated to food quality, expressed as C/N ratio. To explain this mismatch between EPR and food concentration, a series of mixed phytoplankton species dominated by diatoms (≥11 µm filtrate representing natural diatom assemblages: NDA) and 7 single diatom species, all occurring during blooms in the field, were assayed as diets with *C. helgolandicus* females. Ingestion of diatoms by females was estimated by faecal pellet production rates and complementary scanning electron microscopy examinations of diatom remains in pellets. Depending on diatom species in diets, EPR was either increased or depressed 2 to 3 d after food uptake by females had started. The EPR decrease was reversible, when diatom diets were replaced by the dinoflagellate *Prorocentrum minimum*. This effect was also observed when females were transferred to natural phytoplankton populations from the English coast of the Channel close to Plymouth, where food composition in the field differed compared to that off Roscoff. EPR ceased completely when the concentration of NDA diets was artificially increased, but recovered after a shift to a dinoflagellate diet. These results indicate that phytoplankton dominated by diatoms can impair *C. helgolandicus* egg production in the field. This effect was not related to the production of polyunsaturated aldehydes by diatoms. Limitations due to unidentified essential compounds not provided by the metabolism of diatoms, or unknown diatom-derived toxins, were probably involved.

KEY WORDS: Copepod · Diatom · Egg production · English Channel

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INTRODUCTION

Reproduction success of copepods is influenced by many factors, including temperature, food concentration and quality, presence of chemically mediating factors, as well as the gonad maturity of adult females (see review by Mauchline 1998, Niehoff 2003, Hassett 2004, Irigoien 2004). Egg production and hatching rates, as well as naupliar development, are other important factors contributing to the overall population recruitment success. Several recent studies have described the negative effects of diatom-rich diets on

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the hatching success and larval development of copepods, mediated by deleterious polyunsaturated aldehydes (Miralto et al. 1999, Pohnert et al. 2002, Poulet et al. 2003, Adolph et al. 2004, Ianora et al. 2004), but egg production can also vary significantly depending on diet. Patterns of variability in egg production conform to changes in dinoflagellate and microzooplankton biomass (Kleppel et al. 1991), as well as to phytoplankton blooms mostly dominated by diatoms (Runge & Plourde 1996, Meyer- Harms et al. 1999). The varying influence of different algae on these stages of ontogenesis has been documented by several laboratory studies (Ban et al. 1997, Pohnert et al. 2002). Since copepod diets are temporally strongly variable under field conditions, changes in both the egg production and hatching rates can be expected. This point is particularly relevant during the breeding season of *Calanus helgolandicus* in the English Channel, which occurs from early spring to late fall (Pond et al. 1996, Laabir et al. 1998, Irigoien et al. 2000a). In this area, the reproductive season coincides with the succession of diatom blooms, as well as seasonal shifting in species and cell abundances (Grall 1972a,b, Holligan & Harbour 1977, Martin-Jézéquel 1983, Irigoien et al. 2000a,c). Besides the abundant diatom genera *Chaetoceros*, *Rhizosolenia* and *Thalassiosira*, which have been documented in French coastal waters (Beliaeff et al. 2001), *Guinardia* comes to the fore during the summer in coastal waters off Roscoff (Grall 1972b, present work), whereas other genera like *Leptocylindricus* are missing. The complex roles of diatom diets on copepod reproduction have been discussed intensively among the research community concerned with oceanographic plankton. This is above all due to the fact that most evidence showing that certain diatoms can decrease the reproduction capacity of copepods is based on laboratory experiments (see e.g. Poulet et al. 1994, 1995, Ban et al. 1997, Ianora et al. 2004). It has been claimed that these data do not reflect natural diet characteristics, since high diatom concentrations and single-species diets would not be relevant to field conditions, even under bloom situations (Irigoien et al. 2002, Paffenhöfer et al. 2005). Indeed, field work showed that mean per capita egg production increases during spring/summer phytoplankton blooms, but single values over the year oscillate (Niehoff et al. 1999, Irigoien et al. 2000a,b,c).

This observation was also reported from an earlier series of field estimates performed on the British and French sides of the Western English Channel, off Plymouth and Roscoff (Pond et al. 1996, Laabir et al. 1998), and recently in the Eastern English Channel (Devreker et al. 2005).

In bioassays conducted under laboratory conditions, Ban et al. (1997) found, with combinations of different copepod and diatom species, that certain single-species diatom diets can arrest egg production and/or inhibit hatching. Up to now, it has not been possible to elucidate the reason(s) for the EPR (egg production rate) decrease. It might be caused by low female gonad maturation (Niehoff et al. 2002), by toxic diatom-derived aldehydes (Miralto et al. 1999, Pohnert et al. 2002, Poulet et al. 2003, Romano et al. 2003, Adolph et al. 2004, Ianora et al. 2004), or by the low nutritional value of diatoms (Kleppel et al. 1991, Jónasdóttir et al. 1998, Klein Breteler et al. 1999, Hassett 2004, Jones & Flynn 2005). Neither of these concepts can be seen as exclusively valid, since it is difficult to separate the involved factors in laboratory and field assays. But, other factors also have to be taken into account if copepod population success is to be considered. Ohman et al. (2004), among others, have argued that the decrease of a *Calanus* spp. population in the field is exclusively caused by predation and egg cannibalism. Demographic oscillations might thus be caused by intense predation on eggs and juveniles (Ohman et al. 2004). Mortality of nauplii, caused by unsaturated aldehydes like 2,4-decadienal, might also be a limiting factor (Poulet et al. 2003, Ianora et al. 2004), as well as the egg production deficit, which is described in the present paper.

From 1999 to 2002, we observed that the egg production and hatching rates of *Calanus helgolandicus* were surprisingly abnormal from spring to fall at Roscoff (S. A. Poulet unpubl. data), compared to a previous survey (Laabir et al. 1998). Earlier evidence existed on the negative impact of diatom-rich diets on *C. helgolandicus* reproduction (Laabir et al. 1995a, Poulet et al. 1995), but no follow-up studies had been done to address this aspect systematically for the Roscoff area. Thus, we decided to examine in detail the effects of diatoms on the EPR of *C. helgolandicus*. A field survey was carried out during 2003 and 2004 to compare data with the EPR values observed in 1994 (Laabir et al. 1998). We used protocols (Laabir et al. 1995b, 1998), originally developed for the monitoring of EPR in laboratory investigations when feeding natural phytoplankton or single diatoms, to identify the specific influence of different diatom species on *C. helgolandicus* egg production.

This contribution describes the negative influence of diatom-rich phytoplankton on the egg production of *Calanus helgolandicus* in coastal waters off Roscoff, and is the first part of a series of experiments performed to develop an improved understanding of the very variable reproductive success of calanoids. Also based on the data from this survey (2003 and 2004), the effects of polyunsaturated aldehydes and of essential fatty acids on reproductive success will be described in a separate paper by T. Wichard et al. (unpubl. data)

and the morphology and histology of gonads related to EPR decreases among *C. helgolandicus* females will be reported by S. A. Poulet et al. (unpubl. data).

MATERIALS AND METHODS

Pseudo-field experiments. Field estimates from 1994 of the *Calanus helgolandicus* female EPR prevailing at Roscoff were taken from Laabir et al. (1998). The same methods were used in the 2003 and 2004 experiments described here. *C. helgolandicus* specimens were collected several times a week off Roscoff (48° 45' N and 3° 58' W, in the Western English Channel, France), during spring/summer surveys, by towing a 500 µm mesh plankton net obliquely from 20 to 0 m. Samples were transported within 1 to 2 h to the laboratory, where adult, sexually mature females (12 to 30 in total) were sorted for each experiment and were incubated individually for 24 h in dishes containing 100 ml of 0.22 µm filtered seawater, in order to estimate initial EPR, corresponding to field conditions (Laabir et al. 1995a).

Diatom isolation and cultivation. All tested diatoms were successfully isolated from Roscoff plankton samples from March to August in 2004 and cultured in filtered seawater enriched with K-medium at 14°C with a 14:10 h light:dark cycle. Isolation, purification and culture of these key diatom species were achieved according to standard methods (Guillard & Ryther 1962, Keller et al. 1987). The diatoms were identified after Drebes (1974) and Hasle et al. (1996). Three of the tested isolates (TR, GD and RS) are responsible for annual local spring and summer blooms that have been observed in Roscoff waters for decades (Grall 1972a,b, Martin-Jézéquel 1983).

Experiments with single diatom species. To test the effect of single-species diets on EPR at various times during the breeding season, single females were transferred after 24 h to dishes with 80 ml filtered seawater, enriched with 20 ml of diatom culture. The 5 different diatom diets used on different dates in 2004 (Fig. 1C: O) were TR (*Thalassiosira rotula*), RS (*Rhizosolenia setigera*), OR (*Odontella regia*), GD (*Guinardia delicatula:* ex. *Rhizosolenia delicatula*) and GS (*Guinardia striata*), set at final cell densities in the incubators corresponding to 2.5×10^4, 8.25×10^3, 0.6×10^3, 6.58×10^4 and 4×10^4 cells ml^{-1}, respectively. These cell densities were based on the concentration of algae in each culture reaching the exponential growth phase before harvesting. The algal culture was renewed daily during the incubation period, which did not exceed 8 d. EPR values were estimated following the techniques described by Laabir et al. (1995a). In 2003, temperatures during incubations were set at 14 ± 2°C before June and subsequently raised to 20 ± 3°C. In 2004, temperature was set at 14 ± 1°C during all incubations. In 1994, during 2 different periods (Fig. 1A: O), *Calanus helgolandicus* females were assayed with 2 diatom species: TW (*Thalassiosira weissflogii*, Strain RCC 76) and SC (*Skeletonema costatum*, Strain RCC 70) (Laabir et al. 1998).

Experiments with diatom-enriched assemblages. Samples of mixed species in natural diatom assemblages (NDA), collected in 2003 and in 2004 at the same station as the copepod females, were used to test their effect on EPR. Subsurface (1 to 2 m depth) seawater samples were gently filtered by gravity through a filtering column formed of 2 Sartorius filtering funnels, the top one supporting a 350 µm mesh, and the one below, a 11 µm mesh Nitex sieve (Millipore, 45 mm diameter). The top one was used to remove zooplankton and large particles, while the second was used to collect diatoms. Samples corresponding to 200 ml seawater were collected on the 11 µm mesh and re-suspended in incubators containing 100 ml filtered seawater (Millipore, 0.22 µm). Thus, the final concentration of NDA diets in each incubator was approximately 2 times higher than the initial concentration in nature (Table 1). Untreated seawater samples were preserved with Lugol's solution to allow identification of the diatom species in the NDA diets, the ratio between microphytoplankton (<11 µm)/diatom numbers in seawater samples (parallel to NDA 2–NDA 4) and to estimate the cell numbers in the incubators (Table 1). Complementary tests achieved with other NDA diets, set at concentrations corresponding to 2, 10 and 50 times the diatom density in the field, were carried out on several occasions. Besides EPR values in controls with *Prorocentrum minimum* (PM), EPR values with *Calanus helgolandicus* fed on naturally occurring phytoplankton in the field and with NDA diets were compared once during the summer 2003 (see Fig. 4). Cell densities in the enriched NDA samples were comparable to those tested with single-species diets (range: 10^2 to 10^4 cells ml^{-1}). Because copepods can be food limited, this approach allowed us to temporarily increase food biomass and, thus, to incubate females above the food-limitation threshold. New seawater stocks were collected twice a week off Roscoff, at the same station as females, and were used to renew NDA diets every day during the entire incubation period. Particles in these seawater samples, kept in 20 l transparent bottles in the same room as the feeding females, were re-suspended by hand twice a day. In order to obtain a representative spectrum of diatom species occurring successively during the spring/summer blooms, samples for NDA 1, 2, 3 & 4 diets were collected from April to August 2003, and samples for NDA 5, 6 & 7

Table 1. Upper table: Relative proportion and abundance of diatoms in seawater samples collected at a fixed station in the Roscoff coastal zone and used as natural diatom species assemblage (NDA) diets assayed with *Calanus helgolandicus* females in 7 different periods during their breeding season in 2003-2004 (x: species [GD, GS, RS, TR] belonging to 3 genera that were isolated during spring/summer blooms and later used to feed copepods with each single diatom species in diets [same as in Fig. 3]). Lower table: Concentrations of chlorophyll *a*, particulate carbon (POC) and nitrogen (PON) and C/N ratios measured at the same station and same date of sampling as *Calanus* females. Ratio between numbers of microorganisms (<11 µm) and diatoms in preserved seawater samples corresponding to NDA 2, 3 & 4 (see Fig. 4)

Diet:	NDA 1	NDA 2	NDA 3	NDA 4	NDA 5	NDA 6	NDA 7
Date of test:	23/04/ 2003 (%)	07/07/ 2003 (%)	17/07/ 2003 (%)	18/08/ 2003 (%)	23/03/ 2004 (%)	29/03/ 2004 (%)	25/05/ 2004 (%)
Diatom							
Chaetoceros	9.9	13.33	0	50.28	0	7.8	0
Coccinodiscus	0.7	0	0	0	4.5	0	0
Cylindrotheca	0.1	0	0	0	0	0	0
Ditylum	0.3	0	0	0	15.7	7.3	0
Guinardia (x)	31.7	54.3	90.5	20.9	0	0	10.8
Lauderia	8.6	0	0	0	0	0	0
Navicula	0.5	0.32	1.25	0.92	4.5	0.5	0
Nitzschia	0	0	0	0	0		27.3
Pleurosigma	0.1	0	0	0	0	0	0
Pseudo-nitzschia	0.4	0	0	0	11.2	0	0
Rhizosolenia (x)	1.6	16.0	3.8	0.4	12.4	9.1	49.8
Skeletonema	0	8	4.42	20.93	0	0	0
Thalassionema	0.3	8	0	6.52	3.9	69.9	0
Thalassiosira (x)	46.0	0	0	0	36	0.9	12
Total (cells ml^{-1} seawater)	61.28	140.46	73.96	24.84	10.3	12.67	74.96
Diatom abundance in the incubators (cells ml^{-1})	122	281	147.92	49.7	20.6	25.34	149.92
Concentration (µg l^{-1})							
Chlorophyll *a*	1.66	2.05	1.36	1.18	0.8	0.71	1.18
Carbon (POC)	112.8	134.6	113.12	149.2	108.7	100.9	134.9
Nitrogen (PON)	17.9	20.7	17.45	22.7	12.8	13	21.7
C/N ratio	6.3	6.5	6.48	6.57	8.49	7.76	6.21
Ratio of cell numbers							
(in fraction <11 µm)/ diatoms		0.07	0.2	1.18			

were collected from March to May 2004 (see dates of experiment start, Fig. 1, Table 1). According to Grall (1972a), diatoms occurring in Roscoff coastal waters are large (≥8 µm). In agreement with this finding, microscopic observations of NDA diets retained on the 11 µm mesh confirmed that they were dominated by diatoms. We assumed that these NDA diets resembled natural assemblages of diatoms, which the *C. helgolandicus* females should have encountered in the field before capture.

During both 2003 and 2004, the dinoflagellate *Prorocentrum minimum* was used as a control diet (Poulet et al. 1994), at concentrations corresponding to 10^3 to 10^4 cells ml^{-1} in the incubators. The growth conditions for this alga have been described earlier (Poulet et al. 1994). This non-diatom diet was often used to test the reproductive capacity of *Calanus helgolandicus* during the season, thus verifying if low EPR values were linked to sterile females. PM was also used to re-initiate egg production when it had collapsed in the field, or following laboratory treatments with single diatom species or with NDA diets.

For the NDA 1 to NDA 7 diets, the dominant diatom species and dates of the bioassays are given in Table 1. Each bioassay with NDA 1 to NDA 7 was conducted once with a different cohort of carefully selected females (Fig. 1B,C), with undamaged antenna, swimming legs and furca, as well as with a well-maturated genital segment. Bioassays with OR, TR, RS, GD and GS were also performed with carefully selected cohorts of fertile *Calanus helgolandicus* females in 2004 (Fig. 1C).

'Copepod-transfer' experiments. To elucidate the role of naturally occurring diatoms on the female egg production of *Calanus helgolandicus*, 2 parallel incubations were performed at 2 close dates in 2003. While egg production was monitored in Plymouth from 28 August, additional females collected off Plymouth were shipped to Roscoff and incubated there for 3 d in local, untreated seawater, with diatom assemblages resembling one of the NDA 3 or 5 diets (Test T1, Tables 1 & 2). Similarly, on 1 September, females used for the weekly egg production field survey in Roscoff were shipped to Plymouth and incubated there for 5 d with a local non-enriched field diet (Test T2, Table 2). In both assays, following their arrival at Roscoff or Plymouth, females were acclimated 24 h in filtered seawater (Millipore, 0.22 µm) before addition of local phytoplankton food.

Faecal pellet analysis. For each type of diet, ingestion of algal cells by individual copepods was estimated indirectly, through counts of the daily faecal pellet production in each incubator. Moreover, with PM, TR, RS, GD, NDA 1 and NDA 3, faecal pellets were collected daily, during the assays for SEM observations, to verify if the diatoms, identified by their frustule remains, were ingested. The methods used for faecal pellet preparation, SEM examination and documentation have previously been described by Laabir et al. (1995b).

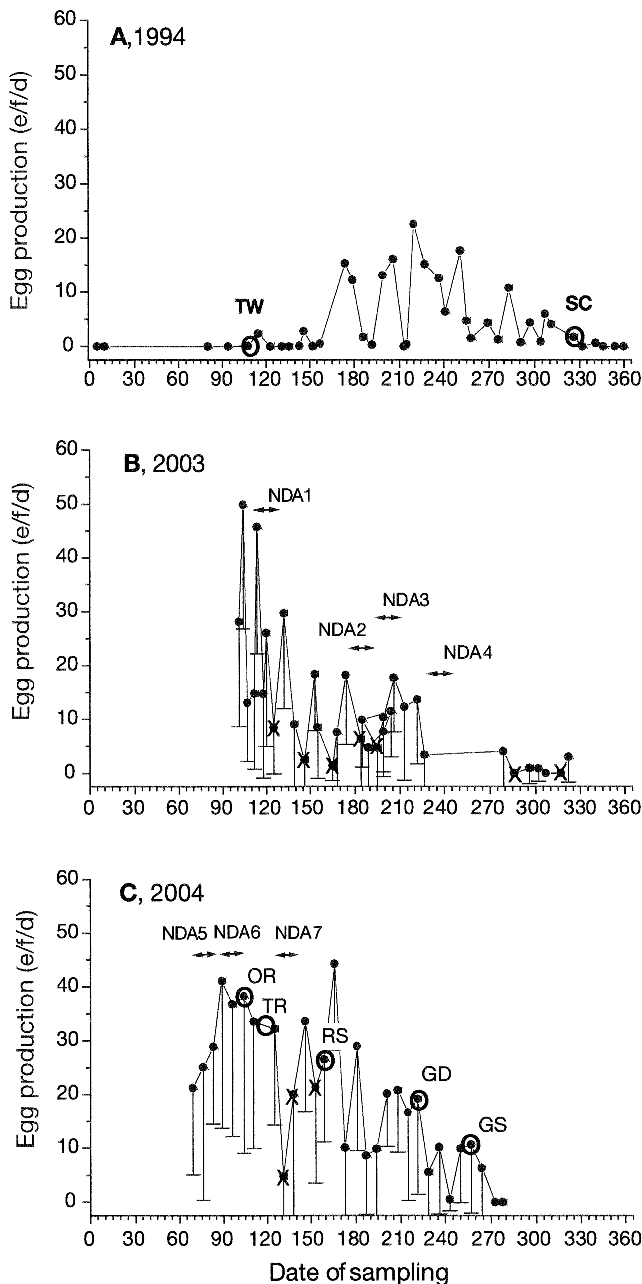


Fig. 1. *Calanus helgolandicus.* Seasonal variation of egg production rates (EPR) in Roscoff coastal waters in (A) 1994, (B) 2003 and (C) 2004. Test periods with natural diatom species assemblages (NDAs, see Fig. 3) are indicated with horizontal arrows. Copepod cohorts fed with NDA 1 to NDA 7 were isolated within this time span. Dates on which EPR induction experiments were performed with single-diatom diets (1994: *Thalassiosira weissflogii*, Strain RCC 76 [TW], *Skeletonema costatum*, Strain RCC 70) [SC]; 2004: *Odontella regia* [OR], *Thalassiosira rotula* [TR], *Rhizosolenia setigera* [RS], *Guinardia delicatula:* ex. *Rhizosolenia delicatula* [GD], *Guinardia striata* [GS]) are indicated with open circles, while dinoflagellates as controls (PM in 2003 and 2004) are indicated with X. Values for 2003 and 2004 are means – SD (bars)

Particulate organic matter and phytoplankton biomass. Every week during 2003 and 2004, untreated subsurface seawater samples were collected at a fixed station off Roscoff at the same time as the *Calanus helgolandicus* females and NDA samples used for the feeding tests. One fraction (250 ml) was preserved with Lugol's solution to determine and evaluate the species and the concentrations of diatoms. Chlorophyll *a* (chl *a*) concentrations were determined by filtering 3 replicate samples (1 l) of seawater from 2 and 60 m depth, collected every 2 wk at the same site as the zooplankton collections, onto GF/F filters and freezing (–30°C) samples for subsequent analyses using a Turner Design fluorometer, according to Yentsch & Menzel's method (1963) and using Lorenzen's (1966) equation. Three further subsamples (1 l) were filtered onto pre-ashed GF/F filters to determine particulate carbon and nitrogen concentrations. Filters were stored at –20°C and then oven dried at 50°C prior to analysis with a Thermo Finnigan CE flash 112 elemental CHN analyser. Only data corresponding to subsurface samples were used in Fig. 2.

RESULTS

The seasonal EPR values of *Calanus helgolandicus* females were extremely variable at Roscoff (Fig. 1). In 1994, peaks of maximum EPR, around 20 to 30 eggs female^{-1} d^{-1}, occurred between the end of May and early August. The rest of the year, lower values were observed. In early spring, autumn and winter, egg production was <10 eggs female^{-1} d^{-1} (Laabir et al. 1998; Fig. 1A). In 2003 and 2004, EPR was evaluated only from March/April to October/November. In contrast to the previous survey, the EPR field estimates were rather different in 2003 and 2004 (Fig. 1B,C). High egg production, with maxima ranging between 30 and 50 eggs female^{-1} d^{-1}, was observed from March to June. The EPR was considerably lower from the end of summer to early fall, with values between 0 and 20 eggs female^{-1} d^{-1} and several drops to 0–5 eggs female^{-1} d^{-1} from August to November. Each year, major oscillations were observed in spring and summer. Mean EPR values (eggs female^{-1} d^{-1} ± SD) were computed for each year from May to November and compared. EPRs were 4.6 ± 4.4 in 1994, 11.9 ± 12 in 2003 and 18.56 ± 13 in 2004. These means were significantly different (*t*-test between years: $t \leq 7.46$, $t > t_{\alpha} = 1.99$, $\alpha = 0.05$, $82 < df < 76$). In 1994, egg production was linearly correlated to phytoplankton biomass determined in the field in terms of chl *a* and particulate organic carbon and nitrogen concentrations (Laabir et al. 1998). In 2003 and 2004, EPR was not significantly correlated to these environmental food factors, to

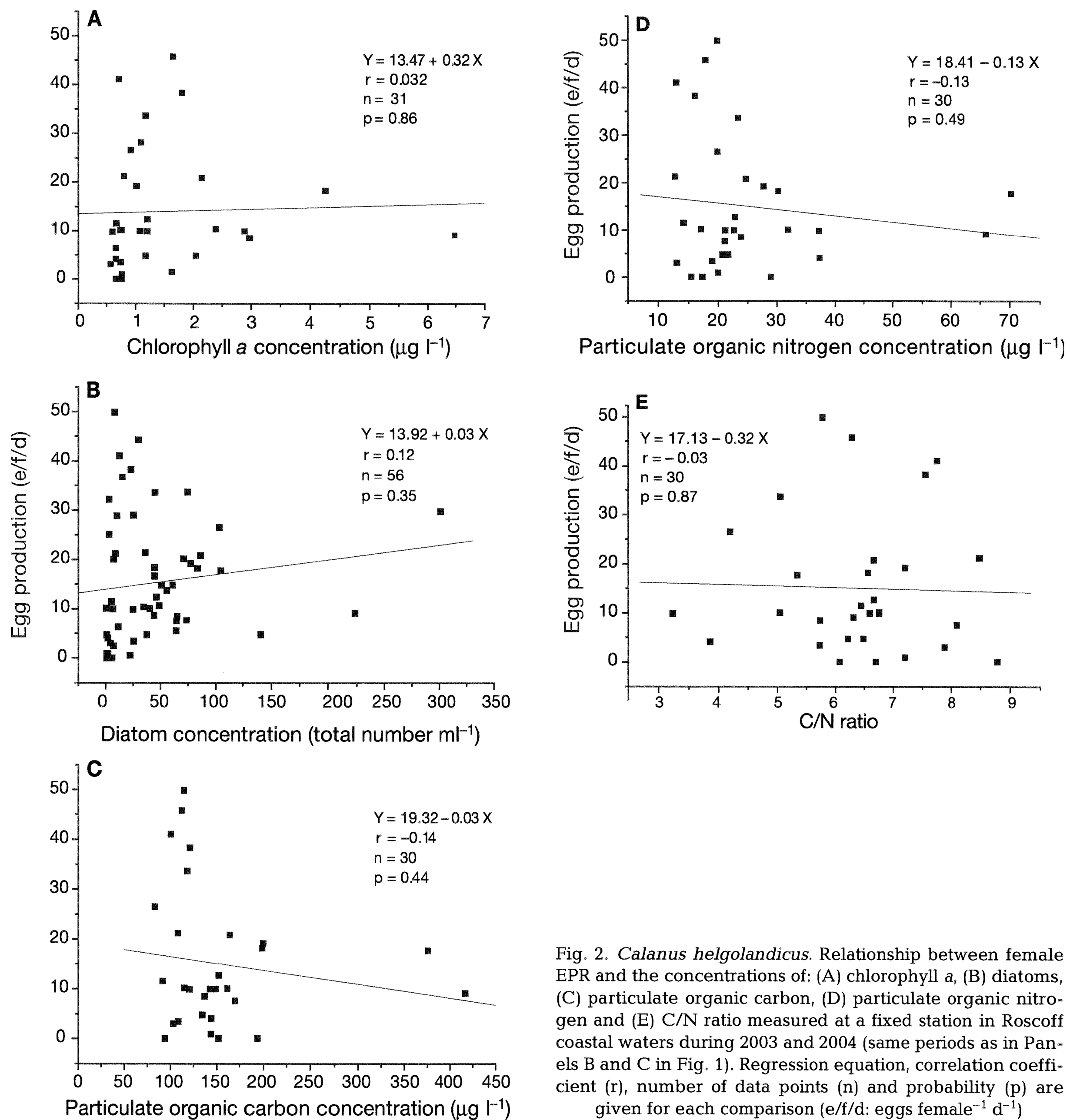


Fig. 2. *Calanus helgolandicus.* Relationship between female EPR and the concentrations of: (A) chlorophyll *a*, (B) diatoms, (C) particulate organic carbon, (D) particulate organic nitrogen and (E) C/N ratio measured at a fixed station in Roscoff coastal waters during 2003 and 2004 (same periods as in Panels B and C in Fig. 1). Regression equation, correlation coefficient (r), number of data points (n) and probability (p) are given for each comparison (e/f/d: eggs female^{-1} d^{-1})

diatom concentrations, or to the C/N ratio, an index of food quality (Fig. 2).

To test the hypothesis that natural assemblages of diatoms can reduce egg production, batches of *Calanus helgolandicus* females belonging to the same cohorts as those used to estimate EPR in the field were exposed to assemblages of mixed diatom species (NDA 1, 2, 3 & 4 in 2003 and NDA 5, 6 & 7 in 2004, see Fig. 1B,C) or to different diets of single diatom species in cultures (TW & SC: see Fig. 1A; TR, RS, OR, GD & GS: see Fig. 1C). A non-diatom diet (PM) was applied as control food. The laboratory tests were performed randomly at times when low or high EPR values had been observed in the field (Fig. 1). Depending on the type of diet, the EPR varied greatly during the incubations. With the natural assemblages of mixed diatom species (NDA 1 to NDA 7; Table 1), the daily mean EPR decreased with time, from 12 to 45 eggs female^{-1} d^{-1} to almost zero, after only 2 or 3 d of incubation (Fig. 3, upper panels). Statistical comparisons of the EPR values between Day 1 and the

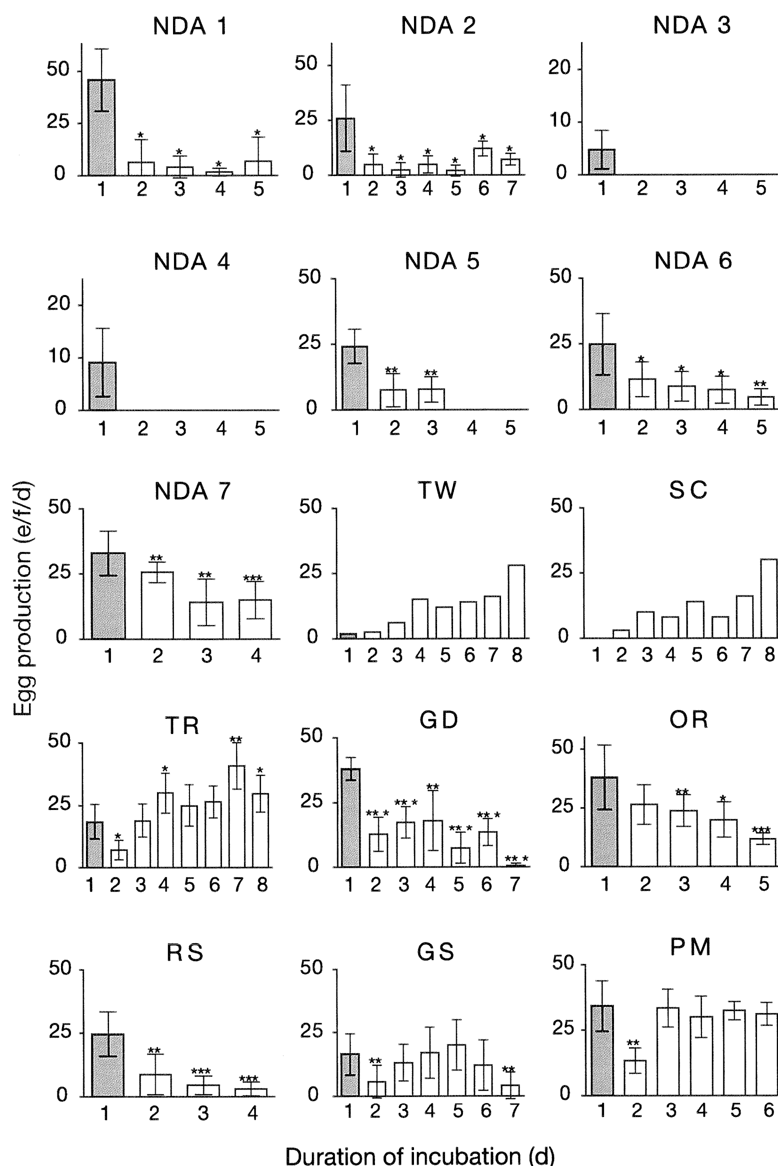


Fig. 3. *Calanus helgolandicus*. Egg production influenced by naturally occurring diatoms. Upper panels: mixed species in NDA 1 to NDA 7 (see composition and abundance of diatoms and corresponding chlorophyll *a*, particulate organic carbon and nitrogen concentrations and C/N ratios in Table 1). Middle and lower panels: isolated species, which were used for feeding experiments. Lower right-hand panel: the non-diatom diet (PM) used as control food. Values on Day 1 (NDA 1 to NDA 7, TW and PM) correspond to initial mean EPR of females estimated in filtered seawater, reflecting EPR in the field (Fig. 1A–C). Values are means ±SD (bars) measured daily in batches of 12 to 20 females per assay. TW and SC were assayed in 1994. NDA 1 to NDA 4 diets were assayed in 2003. NDA 5 to NDA 7 diets were tested in 2004, as well as TR, GD, OR, RS and GS. Error bars based on mean ± 95% CI. Differences of EPR between the initial values on Day 1 (grey bars) and those on the following days with experimental diets were tested with the nonparametric Wilcoxon signed-rank test (significance levels: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Note: this test could not be achieved with the daily mean values for TW and SC borrowed from Laabir et al. (1998), because single daily EPR values for each female in each batch were not available

following days during incubation with NDA 1 to NDA 7 diets indicated that mixed diatoms in NDAs had a significantly unfavourable effect on copepod egg production (Fig. 3, upper panels; non-parametric Wilcoxon rank-signed test, $p < 0.05$). Results in Fig. 4 compare, for the same period (August to September 2003), EPR values between *Calanus helgolandicus* females in nature (field diet) and in bioassays using enriched diatom assemblages (NDA diets) and dinoflagellate cells in controls (PM diet). They showed that EPR values in nature were always above those found with NDA diets, and generally below the values obtained with PM diets. When the biomass of NDA 4 was artificially increased to 2, 10, or 50 times its concentration in the field, the EPR was totally depressed from Day 2 to 5 (Fig. 5). These results support the idea that food quantity was not the limiting factor. With diets of the isolates RS, GD, GS (major summer bloom-forming diatoms) and OR, the EPR values decreased significantly in comparison to initial values on Day 1 as well (Fig. 3; non-parametric Wilcoxon rank-signed test, $p < 0.05$ to 0.001). The diatoms TR, TW and SC induced an EPR increase with time in comparison to the initial values measured on Day 1 (Fig. 3, middle panels). The favourable effect of PM on *C. helgolandicus* reproduction was observed several times over the year in 2003 and 2004 (Fig. 1B,C, cross-labelled data points; and Fig. 3, lower right-hand panel). The same effect has been recognised in other studies (Poulet et al. 1994, Laabir et al. 1995b, Uye 1996, Pohnert et al. 2002). These bioassays suggest that several diatom species exerted different influences on EPR, when offered to *C. helgolandicus* at concentrations close or above bloom conditions. In May 2003, at times when the EPR was around 10 eggs female^{-1} d^{-1} in the field (Fig. 1, e.g. Day 1 in Fig. 6), batches of 20 females, incubated individually under the same conditions, were fed alternately with NDA (1.6×10^2 cells ml^{-1}) and PM (10^4 cells ml^{-1}) diets, during a 12 d incubation period. Egg production was quickly depressed due to the adverse effect of

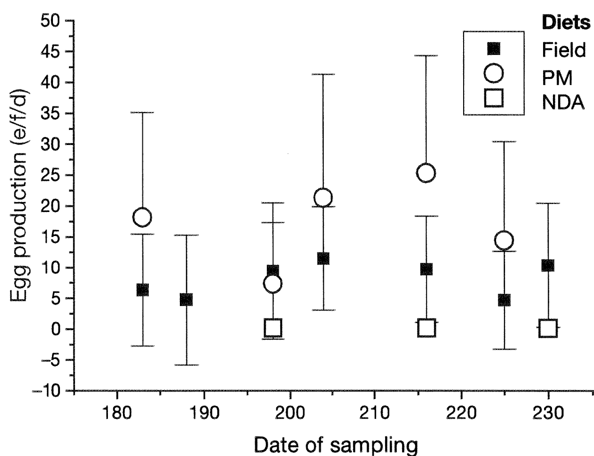


Fig. 4. *Calanus helgolandicus.* Comparison of EPR responses of females to field, PM and NDA diets in July to August 2003. See details in Table 1 for the type of diets, concentration of diatoms and ratio between numbers of microorganisms (<11 µm fraction) and diatoms in seawater-preserved samples corresponding to NDAs 2, 3 & 4

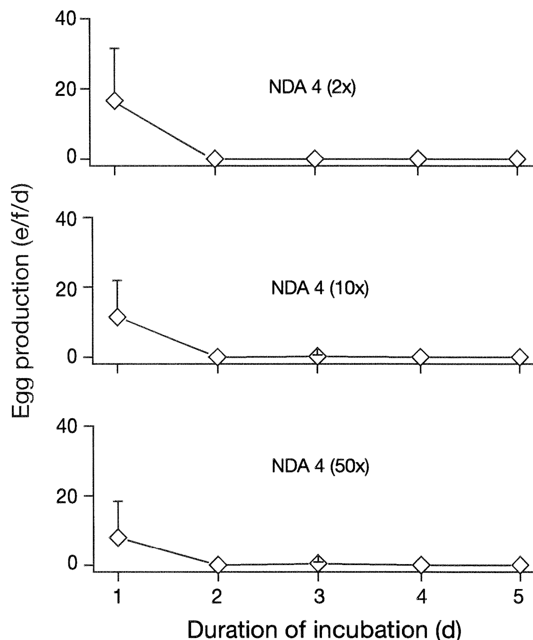


Fig. 5. *Calanus helgolandicus.* EPR responses of females fed the same type of naturally occurring diatoms described for NDA 4 and enriched to 2, 10 and 50 times the field concentration (see Table 1). Values on Day 1 correspond to initial mean EPRs of females in 3 cohorts (10 to 12 females per cohort), belonging to the same population estimated in filtered seawater and reflecting EPR in the field (Fig. 1B). Values are means + SD (bars)

an NDA diet very similar to NDA 1 (Table 1). After the shift of diets, the EPR changed dramatically (Fig. 6), i.e. positive effects with PM and negative effects with NDA were observed repeatedly. The arrest of egg production by NDA was reversible, following a 2 to 3 d period of continuous feeding with the PM diet.

In order to estimate the feeding of *Calanus helgolandicus* on each diet (TW, SC, TR, OR, GD, PM and NDAs), faecal pellet production by females was measured each day for every bioassay. The amount of faecal pellets produced on the respective diets indicates that algae in diets were well ingested by females (Fig. 7). SEM examination of these faecal pellets also showed that the diatoms and non-diatoms (PM) were eaten by the copepods. Remains of TR, RS and GD, mixed in the food with other diatoms, were specifically recognised in the faecal pellets produced under the different NDA diets (see arrows: Fig. 7).

For demonstration of the importance of different diatoms on the reproductive success of *Calanus helgolandicus*, copepod females were exchanged between the biological stations in Roscoff and Plymouth. Two naturally occurring diets in non-enriched seawater samples (Roscoff: T1, Plymouth: T2) reflected the local phytoplankton, particulate matter biomass prevailing in each coastal zone at the time of sampling (Table 2). The main differences between T1 and T2 were a higher chl *a*, POC and PON biomass, a higher density of diatoms and an absence of *Guinardia* spp. in the Plymouth diet (Table 2), which is typical for Plymouth waters (Irigoien et al. 2000a,b). When 'British females', shipped to Roscoff, were exposed to the T1 diet, the EPR decreased sharply from 10 ± 1 to 0 eggs

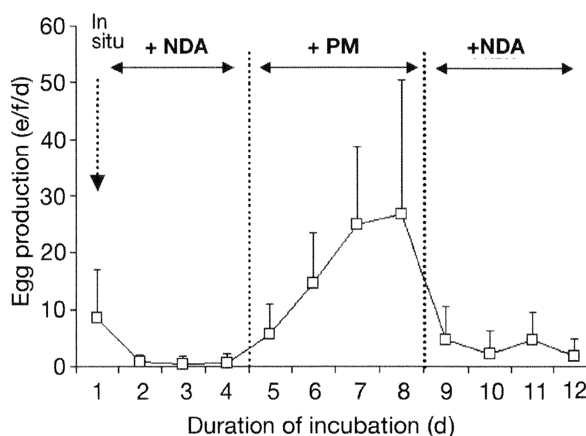
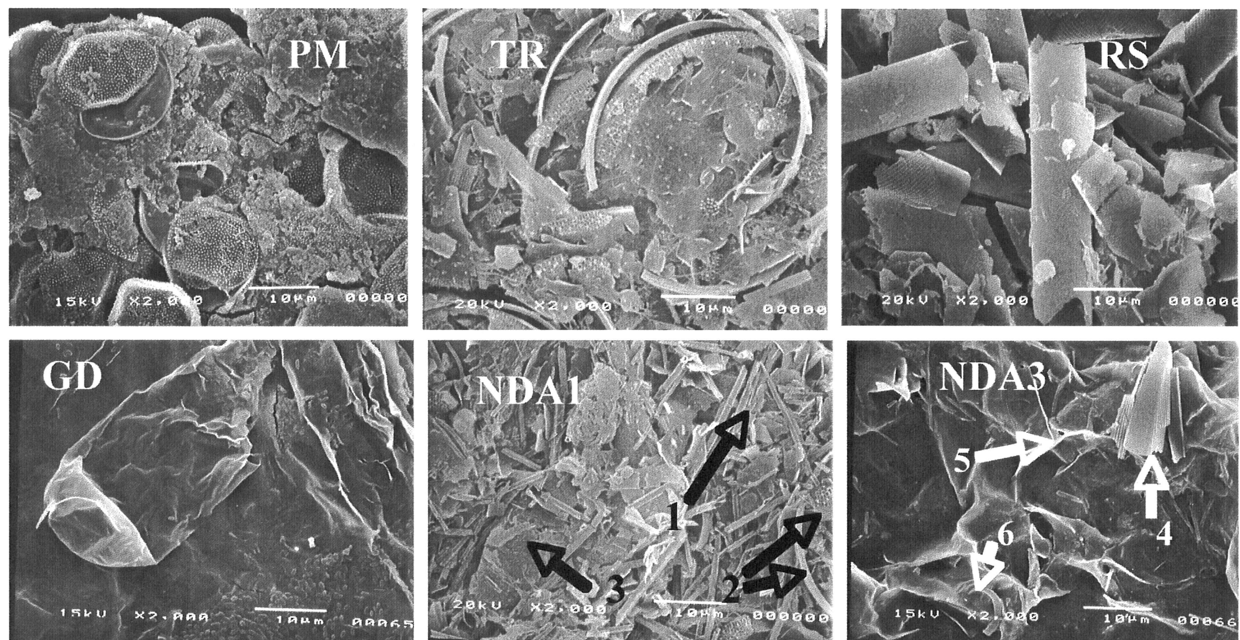


Fig. 6. *Calanus helgolandicus.* Mean daily EPRs with alternating NDA and PM diets. Mean value on Day 1 (5 May 2003) represents estimated rate in filtered seawater, reflecting EPR in the field. Two new NDAs were provided to copepods from Day 1 to 3 and from Day 8 to 12, respectively, alternating with PM provided from Day 4 to 7. The types and concentrations of diatoms in the 2 NDA diets were checked under the microscope and were close to the status of NDA 1 sampled in April (see Table 1). Values are means + SD (bars)



TYPE OF DIET

PM	TW	SC	**TR**	OR	**RS**	**GD**	**NDA1**	**NDA3**
51–94	>20	>20	56–90	25–43	10–87	27–77	2–30	23–105

Faecal pellet production (p/f/d)

Fig. 7. *Calanus helgolandicus.* Daily production of faecal pellets and SEM pictures of algal food remains in copepod faeces. Different single-species diets were tested, as well as natural diatom mixed-species assemblages (NDA 1 & 3 diets: see composition in Table 1). Diatom remains identified in pellets are shown by arrows (1: *Chaetoceros* spp.; 2: *Thalassiosira* spp.; 3: *Guinardia* spp.; 4: *Navicula* spp.; 5: *Guinardia* spp.; 6: *Chaetoceros* spp.). Ranges of daily pellet production per female recorded during the assays (see Fig. 3) are given for 9 different diets. Pellets produced with 6 diets (bold letters) were investigated by scanning electron microscope for species identification (p/f/d: pellets female^{-1} d^{-1})

female^{-1} d^{-1} at the end of the incubation period. In contrast, at Plymouth, the T2 diet led to a slight EPR increase, from 12 ± 13 to 16 ± 7 eggs female^{-1} d^{-1}, along with high hatching rates of 'French females' (Table 3).

DISCUSSION

Over the past 3 decades, the major diatom species causing spring/summer blooms in Roscoff coastal waters have remained the same, including *Guinardia delicatula* (ex *Rhizosolenia delicatula*), *G. striata*, *G. flacida*, *Rhizosolenia setigera*, *Rhizosolenia* spp., *Thalassiosira rotula*, *Chaetoceros* sp. and *Odontella regia* (Grall 1972a, b, Martin-Jézéquel 1983, Gaillard et al. 2002; see Table 1). The increases of chl *a* observed during spring/summer at Roscoff (Waffar et al. 1983, Sournia & Birrien 1995, Laabir et al. 1998, P. Morin unpubl. data) were mainly due to the same diatoms as those that have occurred for decades and matched the species composition observed in the NDA diets in 2003 and 2004 (Table 1).

Observations reported in Fig. 1B,C for 2003 and 2004 reveal that EPR values were highly unstable at Roscoff, resembling data obtained in 1994 by Laabir et al. (1998). However, the seasonal variations showed a major difference between 2003/2004 and 1994. A decade ago *Calanus helgolandicus* was a late spawner (Laabir et al. 1998), while it became an early spawner in 2003 and 2004 (Fig. 1).

This might be explained by different climatic conditions, since 2003 and 2004 were exceptional years in terms of the number of sunny days, mediating higher chlorophyll concentrations during spring/summer (P. Morin unpubl. data). Arrest of egg production in the field has frequently been observed, mainly during fall and winter, corresponding to the lowest chl *a* values (Laabir et al. 1998). Our data now show that this parameter can also be very variable during spring and summer (Fig. 1). The fluctuations of EPR were not correlated to the phyto-

Table 2. Upper table: proportion and abundance of diatom species in seawater samples collected at 2 fixed stations, located in the Plymouth and Roscoff coastal zones, and used as non-enriched diets (see Tests T1 and T2) with *Calanus helgolandicus* females for the trans-Channel parallel bioassays in August to September 2003 (see results in Table 3). Lower table: concentrations of chlorophyll *a*, particulate carbon (POC), nitrogen (PON) and the C/N ratios measured at the same 2 stations

Diatoms	Plymouth 28/08/2003 (T2 diet) (%)	Roscoff 01/09/2003 (T1 diet) (%)
Cerataulina	0.39	0
Chaetoceros	15.59	30.20
Eucampia	0.04	0
Guinardia	0	10.30
Leptocylindricus	27.27	0
Navicula	0	1.52
Nitzschia	48.75	50.42
Rhizosolenia	0.64	0
Roperia	0.004	0
Stauroneis	0.27	0
Thalassionema	1.97	2.89
Thalassiosira	4.77	4.55
Total (cells ml^{-1})	414.15	46.59
Concentration ($\mu g\ l^{-1}$)		
Chlorophyll *a*	0.73	1.21
Carbon (POC)	301.7	152.2
Nitrogen (PON)	43.9	22.8
C/N	6.9	6.1

plankton biomass in the field or to the other biological parameters considered in this work as food factors (Fig. 2). This finding is in accordance with *in situ* observations in the southern Gulf of St. Lawrence (Maps et al. 2005) and in the North Sea (Arendt et al. 2005), where EPR values in *Temora longicornis* did not correlate with chl *a* or temperature; or in Florida Bay, where egg production of *Acartia tonsa* varied independently of the concentrations of proteins, carbohydrates, lipids and most fatty acids in

Table 3. *Calanus helgolandicus.* Reproduction rates of 2 female cohorts used in bioassays in response to different natural food conditions prevailing at 2 stations located on each side of the English Channel (see type of diatoms and phytoplankton biomass in Table 2). Egg production and hatching rates of females at Plymouth (Test 1, 28 August 2003) and Roscoff (Test 2, 1 September 2003) (e/f/d: eggs female^{-1} d^{-1})

	Test 1		Test 2	
	Plymouth (initial)	Roscoff (final)	Roscoff (initial)	Plymouth (final)
Egg production (e/f/d)	10 ± 1	0	12 ± 13	16 ± 7
Hatching (%)	87	?	0	90

the seston (Hazzard & Kleppel 2003). Assuming that only biotic parameters influence the EPR, one should expect optimum EPR values with *Calanus helgolandicus* under high chlorophyll conditions (estimated to be ≥33 eggs female^{-1} d^{-1}: see Mauchline 1998, Ianora et al. 2003). This value is nearly always higher than the EPR observed in the coastal waters off Roscoff (1993, 1994: Laabir et al. 1998; 2003 and 2004: Fig. 1), while a similar discrepancy was detected in the NE Atlantic with *T. longicornis* (Devreker et al. 2005). This phenomenon could be partly due to the age of females, some of which are arrested at the pre- or post-reproductive stages (Niehoff et al. 1999, 2002, Niehoff 2003). However, results with our control tests (PM diet) showed that the sexually mature females were not arrested at pre- or post-reproductive stages, although they only reached 50 to 80% of the optimum specific value of 40 eggs.

The increase of EPRs observed during the early spring in 2003 and in 2004 coincided with diatom assemblages with different dominant species (Table 1). However, even during these periods, short phases of strongly reduced EPRs were observed, which might be attributed to short time shifts in NDAs (Fig. 1B,C). An EPR decrease was observed during late-spring and summer months, coinciding with specific diatom blooms (Table 1, Fig. 3). We suspected that EPR variation might be due to fluctuations in natural diet composition and designed a series of experiments to elucidate the influence of food on egg production. In our bioassays every natural assemblage of mixed diatoms (NDAs), enriched 2 or more times the field concentrations (Figs. 3, 4 & 5, Table 1), as well as some of the isolated individual species (OR, RS, GD, GS), were detrimental to copepod egg production (Figs. 3 & 5). Other diatoms (SC, TR), as well as the dinoflagellate PM, could restore or even increase the EPR (Table 1, Fig. 3). It is interesting that particularly RS and GD, which could be made responsible for reduced EPRs, belong to the spring- to summer-bloom diatoms. Notably all NDA diets (1 to 7) contained GD and RS — albeit not always as major species — whereas the beneficial diet from Plymouth lacked these species (T2 diet; Table 2). Therefore, it is tempting to conclude that the frequent oscillations in the fecundity of *Calanus helgolandicus* females, observed during spring and summer of 2003 and 2004, were linked to successions of a few specific diatoms appearing, or co-occurring during the blooms.

A food effect on fluctuating EPRs would require that the detrimental effects of food are completely reversible under fluctuating food regimes. This reversibility was observed in a laboratory assay in which copepod egg production decreased as the result of a diet of natural diatom assemblages, while it repeatedly recovered when copepods were fed a dinoflagellate diet (PM, Fig. 6). Apparently, the reversible effect illus-

trated in Fig. 6 was not simply due to variation in the cell concentrations between the NDA 1 (2×) and PM diets, set at 1.22×10^2 and 10^4 cells ml^{-1}, respectively. In fact, when NDA 4 (50×) was artificially increased up to 1.25×10^3 cells ml^{-1}, corresponding to a food concentration 1 order of magnitude below PM and 2 orders of magnitude higher than NDA 4 in nature (Day 1), EPR values were consistently depressed (Table 1, Fig. 5). Carbon concentrations in copepod diets were estimated using the values 274 pg $cell^{-1}$ (PM), 624 pg $cell^{-1}$ (TR) and 226 pg $cell^{-1}$ (GD), based on measurements made by Ianora & Poulet (1993) and Wichard et al. (2005). By comparison, the carbon concentrations in the GD and TR diets were 15.6 µg ml^{-1} (with TR), >15 µg ml^{-1} (with GD), and >2.74 µg ml^{-1} (with PM), further suggesting that food biomass was not the limiting factor (Figs. 3, 5 & 6), because TR and GD had inverse effects on EPR. Moreover, the increase of carbon concentration from 0.03 to 0.14 µg ml^{-1} (estimating carbon content of NDA 4 diets equivalent to the cumulated values of cell carbon in each dominant species occurring in NDA 4, Table 1) by increasing the cell concentration (NDA 4: 10× to 50×; Fig. 5) did not result in higher EPR.

Polyunsaturated aldehydes (PUA), produced by several diatoms, have been discussed to be involved in the chemical defence of diatoms, since they have a negative influence on egg and naupliar development (Miralto et al. 1999, Ianora et al. 2004). A recent survey of the production of PUA allowed us to elucidate whether these chemicals may also be responsible for the observed effects, since the isolates used in this work belonged both to PUA producers and non-producers (Wichard et al. 2005). The PUA producers TR and GD had opposite positive and negative effects, respectively. A positive effect was observed for SC (Laabir et al. 1998), also known to be a PUA producer (Wichard et al. 2005). In contrast, OR, which only produces traces of PUA, and 3 non-producers had either a negative (RS, GS), or a positive (TW) effect on EPR. This complete lack of correlation between PUA production and EPR indicates that these metabolites are not an influencing factor in the egg production process. Other chemical factors missing in diets (Hassett 2004), unbalanced diets (Jónasdóttir et al. 1998, Hazzard & Kleppel 2003, Jones & Flynn 2005), or starvation, as caused by fall to winter conditions in the English Channel, might influence copepod population size (Irigoien 2004). But we still do not know which factors in phytoplankton diets determine favourable or less favourable conditions for *Calanus helgolandicus* egg production.

Until now it remains an open question whether the coastal waters off Roscoff are an exceptionally unfavourable site for *Calanus helgolandicus* reproduction. Other sites in the world, apparently, are better for *Calanus* spp. fecundity, such as Plymouth in the Northern Hemisphere (Pond et al. 1996, Laabir et al. 1998, Irigoien et al. 2000a) or the Benguella upwelling system in the Southern Hemisphere (Richardson & Verheye 1999, Richardson et al. 2001). Other ecosystems might be similar or intermediate between the Roscoff and Plymouth cases, for example the North Adriatic Sea (Miralto et al. 1999, 2003, Ianora et al. 2004), or sites where variable spring to summer fecundity and low hatching rate values (0 to 80%) have also been reported (Irigoien et al. 2000b, 2002). The contrasting reproductive responses (EPR and hatching; Table 3) of *C. helgolandicus* between Plymouth and Roscoff were assumed to be due to different hydrological conditions (stratified versus homogeneous water mass) supporting phytoplankton blooms formed of different local species (Holligan & Harbour 1977, Laabir et al. 1998, Irigoien et al. 2000a; Tables 1 & 2; plankton composition in Plymouth can be found at www.pml.ac.uk/L4/). In this context the results of the trans-channel reproduction tests (Tables 2 & 3) allowed us to draw 3 conclusions: (1) the reproductive responses of *C. helgolandicus*, reflecting their past feeding history in the field, were very different between Plymouth and Roscoff at the sampling times in late 2004, even though they were closer at other periods (Irigoien & Harris 2003); (2) the past feeding history had no long-term influence on reproductive success, because French copepod females were able to spawn again after a diet shift corresponding to a new Plymouth food assemblage; (3) these differences were supposedly triggered by different natural diets prevailing in the coastal zones on each side of the English Channel off Roscoff and Plymouth (Tables 1 & 2). Moreover, the elevated reproductive success of the 'French females' at Plymouth was not due to the higher diatom density in Plymouth, because 2× to 50× higher cell densities in NDA diets fed to copepods off Roscoff did not increase EPR (Figs. 3 & 5).

We have not explored the response of *Calanus helgolandicus* reproduction to microorganisms (<11 µm, e.g. dinoflagellates and ciliates, which were removed from the NDA diets) belonging to the microbial food-web. Of course, these small-sized planktonic particles also have a key role in the food transfer to copepods. Interestingly, Gailhard & co-workers (2002) recorded the presence of *Prorocentrum* sp. along with *Rhizosolenia* sp. in the summer months from June to August along the French coast from 1992 to 2000. This natural phytoplankton assemblage could overcome the negative effect of some diatom species in nature, and may explain the differences in EPR values on the starting day and the final day of the feeding experiments with the NDA diets. Different effects of phytoplankton on

EPR could be dependant on the dominant diatom species in the food or on modifications of the proportions of diatom and non-diatom cells. Thus, for example, Kleppel et al. (1991) and Kleppel (1993) might not have observed any negative effects of diatoms, because, either these were not detrimental, like TW, SC and TR (Fig. 2), or negative effects were compensated for by dinoflagellates, ciliates, or other microphytoplankton present at that time. Such a situation prevails in Plymouth coastal waters, where non-diatom organisms are abundant and where reproductive responses were always higher than at Roscoff (Laabir et al. 1998, Irigoien et al. 2000a,b; www.pml.ac.uk/L4/; Table 3). Interestingly, Lacoste et al. (2001), Kang & Poulet (2000), Turner et al. (2001) and Jones & Flynn (2005) have performed tests with mixed diets of diatoms/dinoflagellates and with plankton eggs and larvae in comparison to mono-diets of diatoms; they found improved reproductive success of *Calanus helgolandicus*, *Temora stylifera* and *Arcatia tonsa* fed on the mixed diets, or with eggs and larvae. Whereas Turner et al. (2001) claimed a dilution effect of toxic compounds (e.g. unsaturated aldehydes), Kang & Poulet (2000) and Jones & Flynn (2005) noted the improved nutritional status of mixed-organism diets. First approximations of our results for the summer of 2003 seem ambiguous (Fig. 4); they do not clearly show whether the decreases in egg production observed at Roscoff with both the field and NDA diets, in comparison to the PM diet, were due to food deficiency (i.e. related to the removal of organisms in the <11 µm fraction), or to increased toxicity in the NDA diets enriched with diatoms. During July and August (Fig. 4), proportions of non-diatom organisms (<11 µm; corresponding to flagellates, Gymnodineae, Cryptophyceae and coccolithophorids) over diatoms in seawater samples collected at the same time as females and NDA 2, 3 & 4 (see Table 1) were 0.07, 0.2 and 1.18, respectively. These values might suggest that organisms belonging to the non-diatom diets (<11 µm fraction) were not abundant enough to exert a beneficial effect against the deleterious activity of diatoms.

Other factors, such as UV (Lacuna & Uye 2001), pollutants (Micic et al. 2001), or nitrogen availability (Checkley 1980, Jones & Flynn 2005), also known to affect reproduction in invertebrates, were not investigated as potential causes of fecundity anomalies, but can be excluded partially. Copepod females were not exposed to UV light in the laboratory, and possible seawater pollution did not affect the control diets with PM that systematically induced the recovery of egg production, as shown in Fig. 6. Finally EPR was not correlated to particulate nitrogen concentrations.

In summary, we suggest that the *Calanus helgolandicus* egg production is temporarily depressed due to a food factor, related either to *quantitative* food limitation (mainly during fall and winter, when phytoplankton is scarce: Phase 1 in Fig. 7) or to *qualitative* food limitation or composition. This effect could be caused by the deficiency of essential chemicals (when phytoplankton is abundant or sufficient during spring and summer; Fig. 1; Jónasdóttir et al. 1998, Rey-Rassat et al. 2002, Hazzard & Kleppel 2003, Hassett 2004, Arendt et al. 2005), or the presence of hitherto unidentified chemical inhibitors from diatoms. Our work supports the idea that the biomass of other organisms (e.g. autotrophic/heterotrophic flagellates, dinoflagellates, protistan microzooplankton and meroplankton, zooplankton eggs and larvae) could have a highly beneficial effect on copepod growth and egg production only when their proportions are higher than those of diatoms in diets encountered in the field (Roman & Gauzens 1997, Kang & Poulet 2000, Kang et al. 2000, Lacoste et al. 2001, Turner et al. 2001, Arendt et al. 2005). Thus, *C. helgolandicus* egg production in the field is strongly influenced by the succession of different assemblages and densities of phytoplankton, containing either suitable or unsuitable food, mainly related to diatoms. For all these reasons, females can only occasionally reach, or maintain, their optimum EPR (≥33 eggs $female^{-1}$ d^{-1}) during short favourable periods of the breeding season in Roscoff coastal waters.

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Winter-spring phytoplankton blooms in Dabob Bay, Washington

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Abstract

Scientific investigations in Dabob Bay, Washington State, USA, have been extensive since the early 1960s, but phytoplankton blooms have been studied mostly with regard to chlorophyll concentrations and little is known about the phytoplankton species themselves. Here we provide information on the species present, their abundances during blooms, their contribution to organic carbon concentrations and the ability of some phytoplankton species to produce toxic aldehydes that may impact metazoan grazers.

Multiple blooms of phytoplankton, dominated by diatoms, occurred in the late winter-early spring period, with depth-integrated chlorophyll levels ranging from <20 to 230 mg m^{-2} and peaks in February and April. The major bloom species included *Skeletonema costatum, Thalassiosira* spp. and *Chaetoceros* spp; *Phaeocystis* cf. *pouchetii* occurred in 2002 and 2004. Other taxa or groups of organisms that were sometimes abundant included unidentified small flagellates <10 μm in size and unidentified heterotrophic dinoflagellates. Large diatoms usually comprised most of the cell carbon, but a large, heterotrophic dinoflagellate, identified only as *Gyrodinium* "tear" because of its shape, was a major contributor to the microplankton carbon when present even in small numbers. Five *Thalassiosira* species and *S. costatum* were found to produce polyunsaturated aldehydes (PUA) that are known to affect copepod reproduction and hatching success. Our findings are similar to the few previous studies in the last four decades that included phytoplankton species and suggest long-term similarities and relative stability in the phytoplankton species present and their timing in Dabob Bay.
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Keywords: USA; Washington; Puget Sound; Dabob Bay; Chlorophyll *a*; Species composition; Organic carbon; Diatom aldehydes

1. Introduction

Dabob Bay is a deep, temperate fjord located west of the main basin of Puget Sound, Washington State, USA (Fig. 1). It has been the site of a number of investigations of plankton abundance and production in the past because it is readily accessible and is similar to the local open coastal ocean with regard to the occurrence

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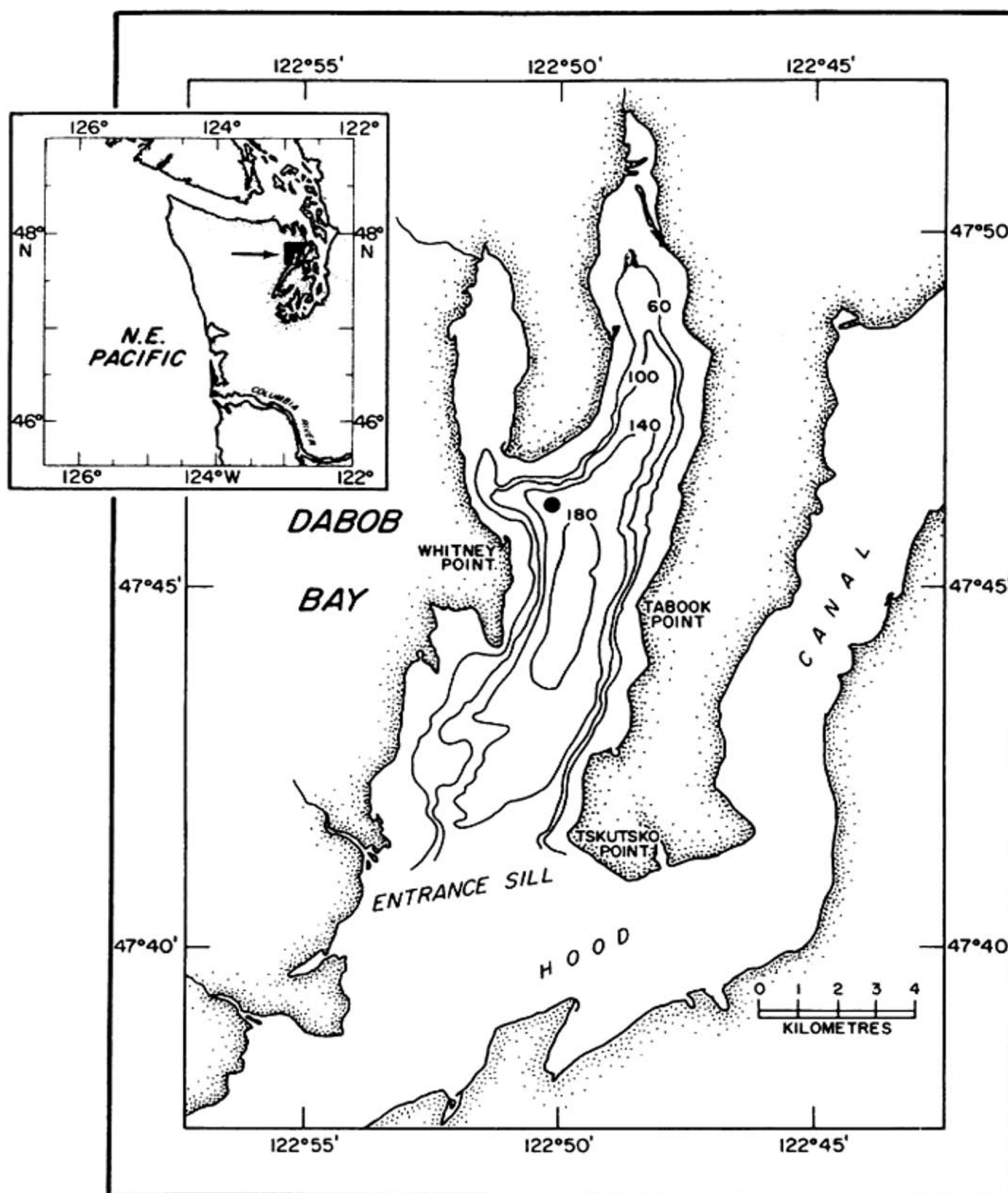


Fig. 1. Sampling location in Dabob Bay, Washington.

and periodicity of phytoplankton blooms and the abundance and composition of zooplankton. Further, the circulation regime, particularly the potentially reduced influence of horizontal advection, is favorable for time series investigations of plankton processes.

Although investigations have been extensive in Dabob Bay since the early 1960s, little is known about the phytoplankton, especially species composition, organic carbon contribution, or possible toxicity to zooplankton because of the production of certain aldehydes. Most of the earlier work has been on zooplankton, including feeding (e.g., Dagg, Frost, & Newton, 1997, 1998; Downs & Lorenzen, 1985; Frost, 1985; Yen, 1982, 1985); reproduction and life cycles (e.g., Ohman & Wood, 1996; Osgood & Frost, 1994; Runge, 1981, 1985); organic matter production (e.g., Bennett, 1980; Copping, 1982; Hedges, Clark, & Cowie, 1988a, 1988b; Prahl et al., 1980; Prahl and Carpenter, 1979); chlorophyll fate and budgets often related to zooplankton grazing (e.g., Buck & Newton, 1995; Dagg, Frost, & Walser, 1989; Shuman, 1978;

Vernet, 1983; Welschmeyer, 1982; Welschmeyer, Copping, Vernet, & Lorenzen, 1984; Welschmeyer & Lorenzen, 1985a, 1985b); seasonal abundance related to advection (e.g., Osgood & Frost, 1996); and physical oceanography (e.g., Ebbesmeyer, 1973; Ebbesmeyer, Barnes, & Langly, 1975; Ebbesmeyer & Barnes, 1980; Kollmeyer, 1965).

With regard to phytoplankton, Larrance (1964) measured chlorophyll *a* and determined species composition approximately twice monthly from February through October 1961. A chlorophyll peak in mid-March was attributed to *Thalassiosira decipiens*, a smaller peak in mid-April contained a green flagellate similar to *Dunaliella* sp., while a peak in mid- to late May was caused by *Chaetoceros concavicornis*. Dortch et al. (1985) studied phytoplankton growth rates in the spring and summer of 1981 and found a bloom in April to be a mixture of pennate and large centric diatoms and *Gonyaulax* sp. In early May, the phytoplankton was dominated by *Chaetoceros* spp., followed by an increasing number of dinoflagellates in mid-May and early July although *Chaetoceros* spp. were still important. An "unidentified, biflagellate green monad" was one of the most numerous organisms during all the cruises. Hedges et al. (1988a) found *Ch. concavicornis* dominated the 65–300 μm size class in the summer and fall of 1981. Other diatoms were also important during the year. In March 1982, *T. decipiens* was dominant, being replaced by *Cerataulina bergonii* in early April. By late April, the most abundant species were the prymnesiophyte *Phaeocystis pouchetii* and the diatom *Nitzschia seriata*. *Skeletonema costatum* and mixed *Chaetoceros* species dominated an early June sample. Postel and Horner (unpublished) sampled twice monthly from June 1997 through February 2001 from a dock located ca. 1 km from the deep-water mooring used for the present work. *Thalassiosira* spp. and *S. costatum* were common and abundant from January through March sometimes extending into April. Other species were often present and numerous, e.g., *Pseudo-nitzschia* spp. in March 1998 and *Dictyocha speculum* and *Ebria tripartita* in February–March 1999. Many large colonies of *Phaeocystis* occurred in April 1999. *Ch. convolutus*, which frequently forms blooms in the fall, was also abundant in January 2000 and February 2001.

This paper reports on the hydrography, chlorophyll *a*, phytoplankton species, and organic carbon from samples collected weekly from February through April in 2002 and 2003 with more limited information from 2004. Data on the ability of certain diatom isolates to produce polyunsaturated aldehydes (PUA) are provided as well. These observations provide the foundation for the following papers on the effects of phytoplankton blooms on grazing, egg production, and naupliar survival of several species of copepods.

2. Materials and methods

Dabob Bay is a long (15 km), narrow (5 km), deep (190 m) fjord, connected over a sill (120 m deep) to Hood Canal, an arm of the Puget Sound system (Fig. 1). The bay is oriented north–south, with the mouth at the southern end open to Hood Canal. There is little tidally induced horizontal motion within the bay (Ebbesmeyer, 1973; Kollmeyer, 1965) and river inflow and estuarine circulation are negligible (Barnes & Ebbesmeyer, 1978), however wind events can have a marked impact on the hydrography (Kollmeyer, 1965). The bathymetry and circulation regime promote containment of both shallow and deep waters within the bay, providing opportunities to test the understanding of time-dependent water column processes such as phytoplankton blooms and zooplankton grazing and vertical migration.

2.1. Field

Twenty-four 2–3 day cruises were carried out during February–April of 2002 and 2003 on board the 65 ft R/V *Clifford A. Barnes*. Two additional cruises occurred in July of both 2002 and 2003 and five cruises between February and April 2004. Cruise dates are given in Table 1.

A CTD/rosette cast was conducted at the beginning of each cruise to determine water properties and the vertical distribution of chlorophyll *a* concentrations and phytoplankton abundances at 12 standard depths (0, 3, 6, 9, 12, 15, 18, 21, 25, 30, 40, 50 m) in the upper 50 m of the water column. The CTD cast, to within 5 m of the bottom (ca. 165 m depth), used a Sea-Bird SBE-25 (2002) or Sea-Bird SBE911 plus (2003), both fitted with a YSI (2002) or Sea-Bird (2003) dissolved oxygen sensor, a WetStar fluorometer, a SeaTech transmissometer, a LiCor PAR sensor, and 12, 10-L Niskin bottles. Additional CTD casts were taken throughout each cruise. Nutrient samples were collected from selected dates and depths. These were frozen until analysis. The chloro-

Table 1
Ship cruise number, dates, and day of the year for cruises in Dabob Bay in 2002–2004

Cruise	Dates	Day of year
737	04–06 Feb 2002	35
738	11–13 Feb	42
739	20–22 Feb	51
740	27 Feb – 01 Mar	58
741	06–08 Mar	65
742	13–15 Mar	72
743	20–22 Mar	79
744	27–29 Mar	86
745	03–05 Apr	93
746	10–11 Apr	100
747	17–18 Apr	107
748	24–25 Apr	114
756	10–11 Jul	191
758	17–18 Jul	198
775	05–06 Feb 2003	36
776	12–13 Feb	43
778	19–20 Feb	50
779	24–25 Feb	55
782	05–06 Mar	64
783	10–12 Mar	69
785	19–20 Mar	78
786	25–26 Mar	84
787	31 Mar – 02 Apr	90
789	09–10 Apr	99
791	17–18 Apr	107
794	30 Apr – 01 May	120
801	10–11 Jul	191
802	16–17 Jul	197
828	21–26 Feb 2004	55
830	03–05 Mar	63
832	10–12 Mar	70
833	22–26 Mar	82
836	07–09 Apr	98

The day of the year is the day of the first CTD cast on each cruise and is used on all figures in this volume.

phyll samples were processed each week and the phytoplankton samples were preserved (see below) and stored for future analyses. The day of the year used in figures here and in the other papers in this volume to distinguish cruises was the day of the first CTD cast on a cruise.

Chlorophyll *a* and phytoplankton samples were also taken from the zooplankton grazing and dilution experiments (Leising, Pierson, Halsband-Lenk, Horner, & Postel, 2005a, 2005b; Leising, Horner, Pierson, Postel, & Halsband-Lenk, 2005c). Water was collected from two layers: above the pycnocline determined from CTD profiles (near surface) and below the pycnocline (deeper) in the upper 25–50 m using the CTD/rosette system. Four depths were selected in each layer based on the profiles of temperature, salinity, and chlorophyll. The depths were evenly spaced over the thickness of the layers, unless there was a distinct peak in the chlorophyll profile, then one depth included that peak. Water was drained from the Niskin bottles into four 9-L polycarbonate carboys. Equal portions from these carboys were then mixed into 20-L carboys. Replicate chlorophyll samples (60–270 ml) from the mixed raw water were filtered onto Whatman GF/F filters during the cruises, immediately placed into 10 ml of 90% acetone, and kept on ice. Replicate ($n = 3$ in 2002, $n = 4$ in 2003) 100–120 ml aliquots of the mixed raw water were preserved with either 1% (v/v) sodium acetate-buffered formalin or acid Lugol's iodine for phytoplankton determinations. These samples provided the time zero concentrations of chlorophyll and cell abundances that grazers would encounter within the designated layers on that day. At the end of each grazing or dilution experiment similar aliquots were collected from the replicate

experiment containers for chlorophyll and phytoplankton determinations. These were filtered or preserved as above. The chlorophyll and phytoplankton samples were analyzed in the shore laboratory (see below).

At the end of each cruise a concentrated phytoplankton sample was collected in a vertical net tow from about 10 m to the surface using a 0.25 m diameter, 20 μm mesh net. The samples were kept alive and stored in a refrigerator for transport to the shore laboratory. These samples provided additional cells for cultures and positive species identifications (see below).

2.2. Laboratory

CTD data were post-processed using Sea-Bird Data Processing software, version 5.25. Temperature and salinity were contour plotted using the Matlab routines in EasyKrig 2.1 software (available from D. Chu, WHOI and the GLOBEC website: http://www.globec.whoi.edu). Nutrient samples were analyzed using a Technicon AutoAnalyzer II system following JGOF protocols (UNESCO, 1994) and plotted as above, but data are not shown here.

Chlorophyll samples were analyzed after 24–48 h additional extraction at −20 °C using fluorometric methods with acidification (Arar & Collins, 1997). The Turner Designs TD-700 laboratory fluorometer was calibrated at the beginning of each field season using chlorophyll *a* standards obtained from Turner Designs. A secondary solid standard, also available from Turner Designs, was used to verify that the calibration did not change prior to each week's sample analysis. Fluorescence readings were made on the original extract of each sample, then three drops of 1.2 N HCl were added and the cuvette was shaken before reading the fluorescence of the acidified extract. Both chlorophyll *a* and the associated phaeopigment concentrations in the water column and experiments were calculated.

Cells in phytoplankton samples from initial controls, final controls, and grazing experiments (see Leising et al., 2005a, 2005b, 2005c) were identified and enumerated using the Utermöhl inverted microscope method (Hasle, 1978). Samples were settled overnight or longer in 50 ml counting chambers. Only the formalin-preserved samples were counted except on days 51, 58 and 86 in 2002 when large flakes of silica, presumably from the walls of the glass jars, made it impossible to count. Then Lugol's preserved samples were counted. All cells in one horizontal transect across the center of the counting chamber were identified and enumerated at 400×, while large, rare cells in one vertical transect across the center of the chamber were counted at 250×. Cells were identified to the lowest possible taxon, usually to species, except for microflagellates that were counted into size classes. When *Phaeocystis* cf. *pouchetii* and *Ch. socialis* were numerous, only the middle field of the chamber was counted with six counts being made and averaged for these species. All cell counts were converted to cells per liter (cells l^{-1}). Three (2002) or four (2003) replicates were analyzed (except see below under calculations). Selected depth profile samples from the Niskin bottles were analyzed as above. References for species identifications included Cupp (1943), Hasle and Syvertsen (1996), Horner (2002), and Steidinger and Tangen (1996).

Live net samples were analyzed within 24 h of collection by placing one or two drops of concentrated cells on a glass microscope slide and examining at 250× or higher magnification using a compound microscope with phase contrast illumination. Species lists were made and relative abundances noted. A subsample from most net tows was preserved in formalin (ca. 1% final concentration) and examined using scanning electron microscopy (SEM) to confirm identifications of *Thalassiosira* spp. Cells from some of the live net tows were isolated for cultures (see below) to be used to determine aldehyde presence or absence.

For SEM, cells from either net tows or counting chambers were rinsed in distilled water to eliminate salt and preservative, acid cleaned (Hasle & Fryxell, 1970), rinsed in distilled water to eliminate the acid, and dried directly on aluminum SEM stubs. The stubs were coated with Au/Pd and examined in a JEOL JSM-840A SEM at an operating voltage of 15 kV.

To determine carbon biomass, some cells of each taxon were measured during counting or from the net tows. Other measurements were taken from our previous work (Horner & Postel, unpublished) or from the literature, e.g., Cupp (1943), Hasle and Syvertsen (1996), Horner (2002). Cell carbon was calculated using the equations in Menden-Deuer and Lessard (2000).

Cultures were started with individual cells or chains isolated from net tows using micropipettes. Cells were rinsed through several changes of f/2 medium (Guillard & Ryther, 1962), placed in individual wells of 12-well

plates, and grown in f/2 medium at 13 °C, 25 μmol photons m^{-2} s^{-1} and 12:12-h L:D cycles. When sufficient growth had occurred in the wells, aliquots were transferred to 125 or 250 ml flasks for additional growth to obtain enough cells for aldehyde analyses. Positive identification of cultured species was made using SEM.

For aldehyde determinations, 20 cultures were sent to the Max Planck Institute for Chemical Ecology, Jena, Germany. The cultures, all in stationary phase, were harvested by filtration (Wichard, Poulet, & Pohnert, 2005b). For a first rapid screening, solid phase microextraction (SPME) was performed with a polydimethylsiloxan fiber after wounding by sonication (Pohnert et al., 2002). To quantify polyunsaturated aldehydes (PUA) released upon cell damage, a protocol based on derivatization of PUA with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamin hydrochloride (PFBHA-HCl) and subsequent GC/MS (EI) analysis was applied (Wichard et al., 2005b). The limit of quantification for PUA in concentrated diatom cultures was 5 ng ml^{-1} and all analyses were performed in triplicate (Wichard et al., 2005a).

2.3. Calculations

About 100 phytoplankton species or taxonomic categories occurred in our samples (Table 2). However, many of these were present only in small numbers or on few occasions. As a result, we have chosen to present detailed data on only nine species or categories of cells (Table 3) for both cell numbers and cell carbon. These were present as the highest numbers, highest carbon, or were of special interest in the grazing experiments, e.g., *Thalassiosira* spp. and *Gyrodinium* "tear." The Other category consists of flagellates from various taxonomic groups, ciliated protists, and amoebae. The flagellates include cryptomonads, choanoflagellates, euglenoids, the chrysophyte *Dinobryon* spp., silicoflagellates (*Dictyocha* spp.), and the ebriid, *Ebria tripartita*. Although our focus was primarily on autotrophs, i.e., the phytoplankton, we have included some heterotrophs, e.g., heterotrophic dinoflagellates, flagellates, ciliates and amoebae in our calculations because they were an important part of the copepod prey (Leising et al., 2005a, 2005b). Moreover, *Gyrodinium* "tear" grazed heavily on *Thalassiosira pacifica* (Fig. 2), one of the prime food items for the copepods and also a major producer of potentially harmful PUA. Cyanobacteria, although known to be present in Dabob Bay, have not been included here because they are not part of the diet of the copepods we studied.

In most cases, the highest cell number of a species or taxonomic category from the three (2002) or four (2003) replicates from the surface initial sample was used for the calculations. In two cases only one sample was available (days 35, 58 in 2002). In one case (day 50 in 2003) the cruise was aborted after the CTD cast was taken and cell counts from the depth profile were used to estimate average cell concentrations. This was done by averaging the cell numbers from the nine samples between 0 and 25 m for the surface layer concentrations and the four samples from 25 to 50 m for the subsurface (deep) layer concentrations.

3. Results

3.1. Hydrography

In general, during the first part of the season in both years, the water column was colder and fresher in the surface layer (0–10 m) than in the deeper subsurface layer, with both layers becoming warmer in early April. There were some differences between years in overall temperature and salinity patterns (Fig. 3).

In 2002, water above 10 m depth warmed from ~7 °C in early February to >11 °C at the end of April. By July this layer was 14–20 °C. In the 10–50 m depth range temperatures were usually 8.5–9.5 °C, relatively uniform over the 40 m thick zone each week, but slightly different in temperature from week to week. This deeper water cooled slightly between early February and early March, then slowly warmed through April and into July when temperatures at these depths exceeded 10 °C. The salinity ranged from 25.5 to 29.0 above 8 m for all cruises in 2002, and slowly increased from 29.3–29.6 to 29.8–30.0 between 15 and 50 m depth.

The 2003 temperature between 10 and 50 m was fairly uniform from February through April, ranging from about 9.0 to 9.3 °C. Above 10 m the range was 8–9.5 °C from early February to late March, then the upper 5 m of water warmed to 11 °C by mid-April and >12 °C in late April, again approaching 20 °C in July. The 5–10 m zone was 2–3 °C colder in 2002 than it was in 2003. The 2003 salinities varied from ~24.3 to 29.5 in the upper 8–10 m from February to April, and then increased from 29.1–29.8 at 15 m to 29.7–30.4 at 50 m.

Table 2
Phytoplankton and protist species found in Dabob Bay, Washington, in the winter-springs of 2002–2004

Diatoms
Actinoptychus senarius (Ehrenberg) Ehrenberg
Asterionellopsis glacialis (Castracane) Round
Bacillaria paxillifer (O.F. Müller) Hendey
Cerataulina pelagica (Cleve) Hendey
Chaetoceros contortus Schütt
Chaetoceros convolutus Castracane
Chaetoceros danicus Cleve
Chaetoceros debilis Cleve
Chaetoceros decipiens Cleve
Chaetoceros diadema (Ehrenberg) Gran
Chaetoceros didymus Ehrenberg
Chaetoceros laciniosus Grunow
Chaetoceros radicans Schütt
Chaetoceros similis Cleve
Chaetoceros socialis Lauder
Chaetoceros subtilis Cleve
Chaetoceros teres Cleve
Chaetoceros vanheurckii Gran
Chaetoceros spp.
Corethron hystrix Hensen
Coscinodiscus centralis Ehrenberg
Coscinodiscus wailesii Gran & Angst
Coscinodiscus spp.
Cylindrotheca closterium (Ehrenberg) Lewin & Reimann
Dactyliosolen blavyanus (H. Peragallo) Hasle
Dactyliosolen fragilissimus (Bergon) Hasle
Ditylum brightwellii (West) Grunow
Guinardia delicatula (Cleve) Hasle
Gyro-Pleurosigma spp.[a]
Leptocylindrus danicus Cleve
Leptocylindrus minimus Gran
Melosira spp.
Navicula spp.
Odontella aurita (Lyngbye) C.A. Agardh
Odontella longicruris (Greville) Hoban
Paralia sulcata (Ehrenberg) Cleve
Pseudo-nitzschia pungens/multiseries
Pseudo-nitzschia pseudodelicatissima (Hasle) Hasle
Pseudo-nitzschia spp.
Rhizosolenia setigera Brightwell
Skeletonema costatum (Greville) Cleve
Stephanopyxis nipponica Gran & Yendo
Thalassionema nitzschioides (Grunow) Grunow
Thalassiosira aestivalis Gran & Angst
Thalassiosira anguste-lineata (A. Schmidt) Fryxell & Hasle
Thalassiosira eccentrica (Ehrenberg) Cleve
Thalassiosira nordenskioeldii Cleve
Thalassiosira pacifica Gran & Angst
Thalassiosira punctigera (Castracane) Hasle
Thalassiosira rotula Meunier
Thalassiosira spp.
Unidentified pennate diatoms
Unidentified centric diatoms

Dinoflagellates
Alexandrium spp.
Amphidinium spp.
Amylax triacantha (Jørgensen) Sournia
Ceratium lineatum (Ehrenberg) Cleve

Table 2 (*continued*)

Dicroerisma psilonereiella Taylor & Cattell
Dinophysis acuminata Claparède & Lachmann
Dinophysis spp.
Gonyaulax spp.
Gyrodinium spirale (Bergh) Kofoid & Swezy
Gyrodinium spp.
Gyrodinium "tear" (see Buck and Newton, 1995)
Heterocapsa triquetra (Ehrenberg) Stein
Minuscula bipes (Paulsen) Lebour
Noctiluca scintillans (Macartney) Kofoid & Swezy
Prorocentrum cf. *minimum* (Pavillard) Schiller
Protoperidinium brevipes (Paulsen) Balech
Protoperidinium conicum (Gran) Balech
Protoperidinium depressum (Bailey) Balech
Protoperidinium oceanicum (VanHöffen) Balech
Protoperidinium steinii (Jørgensen) Balech
Protoperidinium spp.
Scrippsiella trochoidea (Stein) Loeblich III
Unidentified autotrophic dinoflagellates
Unidentified heterotrophic dinoflagellates

Flagellates
Calycomonas gracilis Lohmann
Choanoflagellates
Cryptomonads
Dictyocha speculum Ehrenberg
Dinobryon spp.
Ebria tripartita (Schumann) Lemmermann
Euglenoids
Meringosphaera mediterranea Lohmann
Parvicorbicula socialis (Meunier) Deflandre
Phaeocystis cf. *pouchetii* (Hariot) Lagerheim
Unidentified flagellates <10 μm
Unidentified flagellates 10–20 μm
Unidentified flagellates >20 μm

Ciliates
Laboea conica Lohmann
Unidentified ciliates

Tintinnids
Heliocostomella sp.
Salpingella curta Kofoid & Campbell
Tintinnus pectinis Kofoid & Campbell
Unidentified tintinnids

Amoebae
Paulinella ovalis (Wulff) Johnson, Hargraves & Sieberth
Unidentified large lobose amoebae

[a] *Gyrosigma* spp. and *Pleurosigma* spp. are combined here because of the difficulty in distinguishing them in counting chambers.

In 2002 the water column in Dabob Bay was somewhat less stratified than it was in 2003 based on the σ_t isopycnals over time (data not shown). In February–April 2002, σ_t at 2 m depth ranged from 20.2 to ~21.4 kg m^{-3}, the 22.0 kg m^{-3} isopycnal was located at depths between 4 and 18 m, and water between 25 and 50 m depth maintained a consistent σ_t value of about 23.0–23.2 kg m^{-3} throughout the entire three months. On each cruise σ_t increased $\leqslant$ 0.1 kg m^{-3} between 25 and 50 m. In 2003 σ_t at 2 m depth ranged from 18.3 to 21.8, the 22.0 kg m^{-3} isopycnal was located at depths between 4 and 10 m, and water between 25 and 50 m depth varied from <22.8 to >23.2 kg m^{-3} during the cruises and was slightly different each week. Below 15–50 m the σ_t profile monotonically increased about 0.1–0.2 kg m^{-3}, and the maximum σ_t at 50 m ranged from ca. 22.8 to 23.5 kg m^{-3} in 2003. The σ_t data from 2004 cruises indicated that year was similar in pattern to 2002.

Table 3
Species or categories of taxa used in calculations and figures

Chaetoceros spp. (includes all *Chaetoceros* species)
Thalassiosira spp. (includes all *Thalassiosira* species)
Other diatoms (includes all other diatom species)
Gyrodinium "tear"
Heterotrophic dinoflagellates
Autotrophic dinoflagellates
Flagellates < 10 μm
Phaeocystis cf. *pouchetii*
Other (includes all other taxa or categories, mostly flagellates and ciliates)

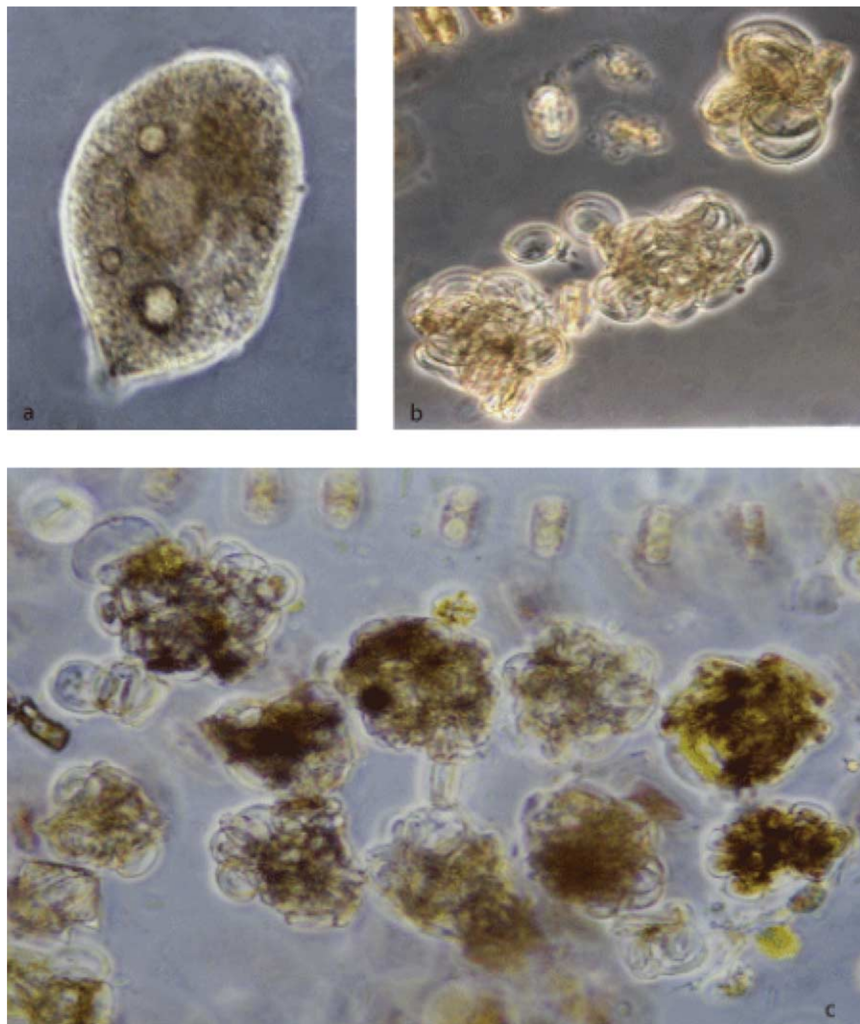


Fig. 2. Light micrographs of *Gyrodinium* "tear" and its fecal pellets containing empty frustules of *Thalassiosira pacifica*. (a) Whole cell without food; cell size: 120 μm long by 72 μm wide; (b and c) fecal pellets; size range: 100–150 μm; *Thalassiosira pacifica* cell frustules inside fecal pellets ca. 60 μm in diameter. All pictures taken at 200× magnification.

Dissolved inorganic nutrient concentrations were not determined every week (not before day 65 in 2002 and only on days 36, 55, 69, 78, and 120 in 2003). In 2002 combined nitrate, nitrite and ammonia surface concentrations were 5.5–21 μM in March, and <0.5 μM in April, while the combined concentrations at 6 m ranged

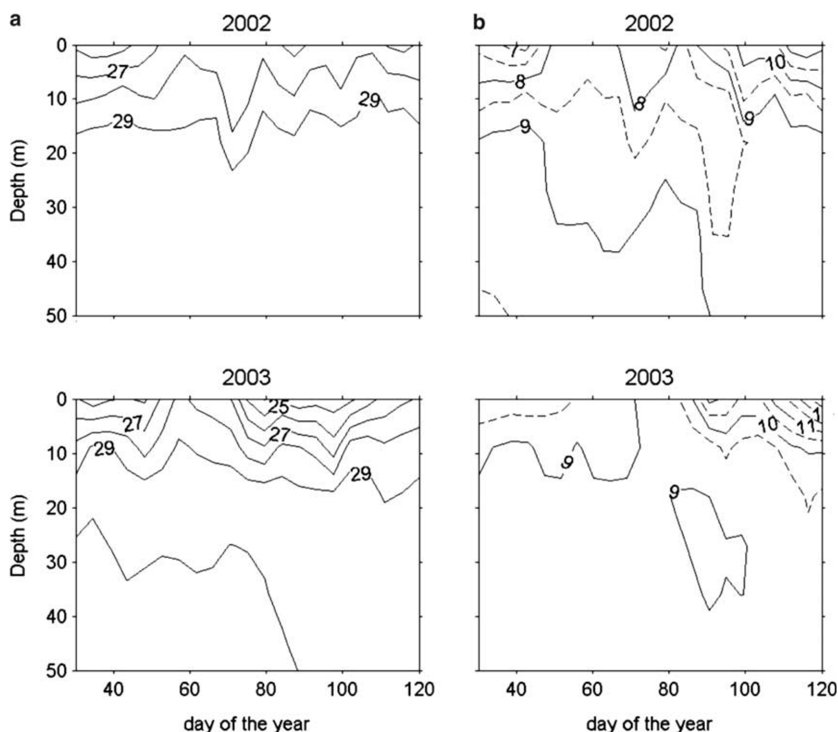


Fig. 3. Water properties in Dabob Bay, 2002 and 2003. (a) Salinity (psu); (b) temperature (°C).

from <0.2 to >24 µM on the same dates. In July 2002 the combined nitrogen concentrations were low (<2.5 µM) in the upper 6–10 m on both cruises. In 2003 the combined concentrations at the surface were again <0.5 µM at the end of April, but ranged from ~4 to >23.5 µM on four cruises where samples were analyzed in February and March. Phosphate levels were >0.4 µM at the surface on all dates sampled between 6 March and 24 April 2002 and on all dates in 2003 until the end of April (day 120) when it dipped to 0.2 µM. Silicate remained above 15 µM in both years except on day 120 in 2003 when it was 4 µM at the surface. In July 2002, phosphate concentrations were <1.0 µM while the corresponding silicate values were 15–20 µM in the upper 6–10 m on both cruises.

3.2. Chlorophyll

During both 2002 and 2003, multiple phytoplankton blooms occurred during the winter-spring period (Fig. 4). In 2002 (Fig. 4(a)), a chlorophyll peak, near 120 mg m^{-2} integrated to 30 m, occurred in mid-February (day 51) dominated by diatoms (see below). This was followed by a drop to mid-winter conditions, <20 mg m^{-2}, that lasted for four weeks. Another increase to near 130 mg m^{-2} occurred in late March (day 86) lasting for nearly three weeks before a second brief decrease to ca. 60 mg m^{-2}. The last peak, the highest of the spring at 230 mg m^{-2}, occurred in late April (day 114) when *Phaeocystis* cf. *pouchetii* was the dominant phytoplankton species.

In 2003 (Fig. 4(b)), a chlorophyll peak near 220 mg m^{-2}, dominated by diatoms, occurred in mid-February (day 50). This was followed by a decrease a week later (day 55) and a smaller increase to ca. 110 mg m^{-2} in early March (day 64). Winter conditions near 20 mg m^{-2} were present in mid-March (days 69, 78). The highest chlorophyll concentration of 230 mg m^{-2} occurred in early April (day 99) after a gradual increase from the winter low. A final peak near 220 mg m^{-2} occurred the end of April (day 120). Diatoms, including *Thalassiosira* spp., *Chaetoceros* spp. and other diatoms, were the dominant phytoplankton species present. In both 2002 and 2003, peaks in depth-integrated phaeopigments between 0 and 30 m occurred after the chlorophyll increased, and were higher in April than in February or March.

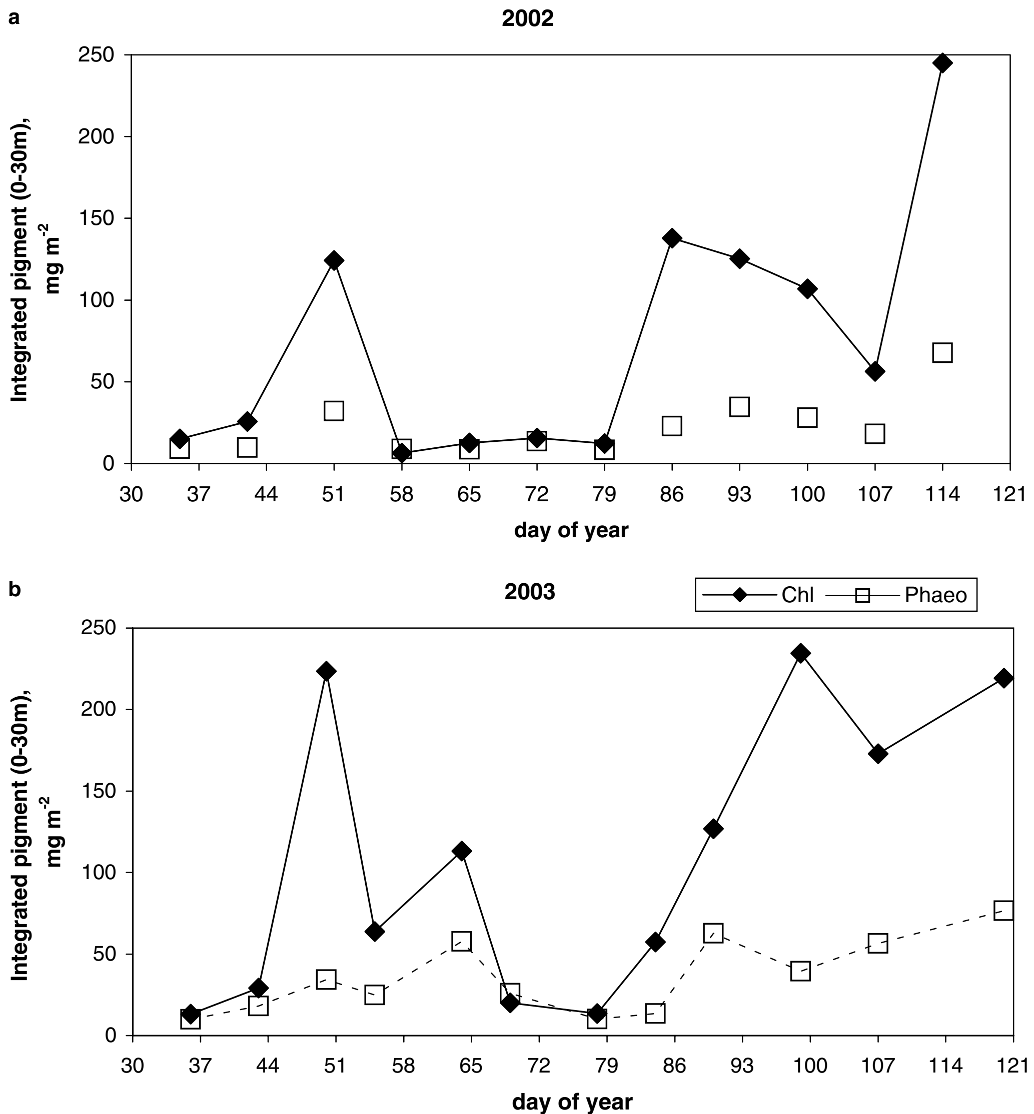


Fig. 4. Depth-integrated chlorophyll and phaeopigments in the upper 30 m. (a) 2002; (b) 2003.

3.3. Phytoplankton species and abundances

Species and other taxonomic categories observed during this study are listed in Table 2 and abundances of major groups in Table 4. Sampling in 2002 started in early February (day 35; Fig. 5(a)). In the surface layer (generally above 12 m), a diatom bloom was already in progress consisting primarily of *S. costatum* and *Thalassiosira* spp., including *T. aestivalis* and *T. pacifica* (identifications confirmed with SEM). The *Thalassiosira* bloom lasted until nearly the end of February (day 51), with *T. aestivalis* reaching 3.3×10^5 cells l^{-1} and *T. pacifica* 4.4×10^5 cells l^{-1}. From the end of February until nearly the end of March (days 58, 65, 72, 79), both phytoplankton cell numbers and chlorophyll were low, and no samples were counted for days 65 and 72. The elevated fraction (>40%) for the Other category on day 58 was attributable to $>1 \times 10^5$ cells l^{-1} of small cryptomonads, while diatoms decreased by nearly two orders of magnitude from the previous week. *Thalassiosira* species returned for two weeks in late March to early April (days 86, 93) with *T. pacifica* reaching nearly 1.5×10^5 cells l^{-1}. Other *Thalassiosira* species present included *T. aestivalis*, *T. anguste-lineata*, and *T. nordenskioeldii*. Total *Thalassiosira* cell numbers were $3.3–6.3 \times 10^5$ cells l^{-1}. By late March, a bloom of *Ch. socialis*

Table 4
Abundance (cells l^{-1}) for the nine species and taxonomic categories listed in Table 3 and used in Fig. 5

A												
Day of year	35	43	51	58	65	72	79	86	93	100	107	114
Species												
Other	8900	100,500	270,700	157,600	na	na	57,300	207,300	33,500	45,000	70,000	82,700
Phaeo	0	0	0	0	na	na	2000	265,500	86,300	699,900	6,783,400	3,338,800
Flg < 10	69800	207,300	83700	142,900	na	na	137,800	285,900	78,100	27,100	110,300	79,100
Autdn	0	1000	3100	1200	na	na	0	0	12,000	44,200	4100	12,700
Hetdn	400	8400	13,700	19,500	na	na	23,300	20,000	24,800	69,700	43,800	117,000
GyroT	0	200	4100	600	na	na	0	0	3500	2000	600	600
Otherdiatoms	60,500	44,300	63,300	12,800	na	na	12,800	97,100	51,400	80,300	64,200	45,700
Thspp	33,600	131,800	793,800	9000	na	na	2000	331,800	628,400	27,300	26,500	14,900
Chspp	2400	8300	24,900	2000	na	na	2000	1,932,800	1,408,800	1,421,800	397,600	103,600
B												
Species												
Other	2600	11,800	63,800	12,600	na	na	25,100	93,100	24,000	20,100	7800	24,100
Phaeo	0	0	0	0	na	na	0	74,500	45,900	238,900	385,900	3,495,500
Flg < 10	11,600	41,200	115,400	33,700	na	na	54,100	201,100	116,400	68,400	93,900	185,800
Autdn	300	0	1200	400	na	na	0	2000	2200	1000	1000	3800
Hetdn	4400	10,400	55,200	5700	na	na	17,100	26,100	22,000	36,000	27,800	37,900
GyroT	0	0	0	200	na	na	1000	0	0	600	1000	600
Otherdiatoms	5300	15,100	18,800	14,000	na	na	1600	24,200	26,700	10,200	9500	29,200
Thspp	3200	14,500	28,100	4700	na	na	2000	184,900	133,800	21,400	4100	5700
Chspp	0	300	400	200	na	na	0	572,800	1,216,000	665,700	126,600	72,600
C												
Day of year	36	43	50	55	64	69	78	84	90	99	107	120
Species												
Other	13,700	39,200	67,500	36,300	61,000	62,900	27,400	122,400	259,700	41,200	34,100	26,400
Phaeo	0	0	1600	0	8200	7100	0	0	2600	4600	0	0
Flg < 10	120,500	257,300	227,300	233,800	345,100	173,600	112,300	162,300	158,800	88,800	73,000	50,500
Autdn	0	2300	100	1000	5700	1600	3200	2600	2900	15,800	13,300	10,800
Hetdn	13,300	22,600	18,000	18,400	62,100	28,200	23,000	28,700	62,400	73,500	66,100	93,900
GyroT	0	0	600	1200	2000	2000	0	0	1800	1000	600	0
Otherdiatoms	72,500	45,100	5800	19,400	36,900	15,500	33,700	204,800	140,500	234,600	489,000	790,200
Thspp	10,200	59,900	202,000	269,400	82,600	9600	6400	7300	260,300	158,700	147,800	111,300
Chspp	7100	48,300	12,200	29,800	218,100	206,800	63,300	97,100	322,600	428,800	566,100	630,000

(*continued on next page*)

Table 4 (*continued*)

D												
Species												
Other	11,800	5700	3200	10,900	9300	13,300	13,400	17,900	21,000	33,500	25,800	na
Phaeo	0	0	0	0	1000	0	0	0	0	0	0	na
Flg < 10	41,200	53,100	12,300	69,400	79,600	66,400	37,800	31,700	47,000	52,100	64,300	na
Autdn	0	0	0	0	0	0	0	0	0	1000	1000	na
Hetdn	10,400	6100	8700	16,300	28,200	23,000	14,900	15,300	14,400	21,900	38,400	na
GyroT	0	0	0	0	1000	2000	0	600	0	0	0	na
Otherdiatoms	15,100	24,200	2700	25,200	7000	2200	14,300	9800	15,500	30,900	59,900	na
Thspp	14,500	13,300	14,500	10,200	15,300	1600	1000	0	3100	12,500	22,500	na
Chspp	300	2000	0	1800	9500	19,400	8500	11,400	4700	115,700	279,800	na

Numbers are the highest of three (2002) or four (2003) replicates. A. surface 2002; B. deep 2002; C. surface 2003; B. deep 2003.
Other = all other species; Phaeo = *Phaeocystis*; Flg10 = flagellates < 10 μm; Autdn = autotrophic dinoflagellates; Hetdn = heterotrophic dinoflagellates; GyroT = *Gyrodinium* "tear"; Otherdiatoms = all other diatoms; Thspp = all *Thalassiosira* species; Chspp = all *Chaetoceros* species; na = not available.

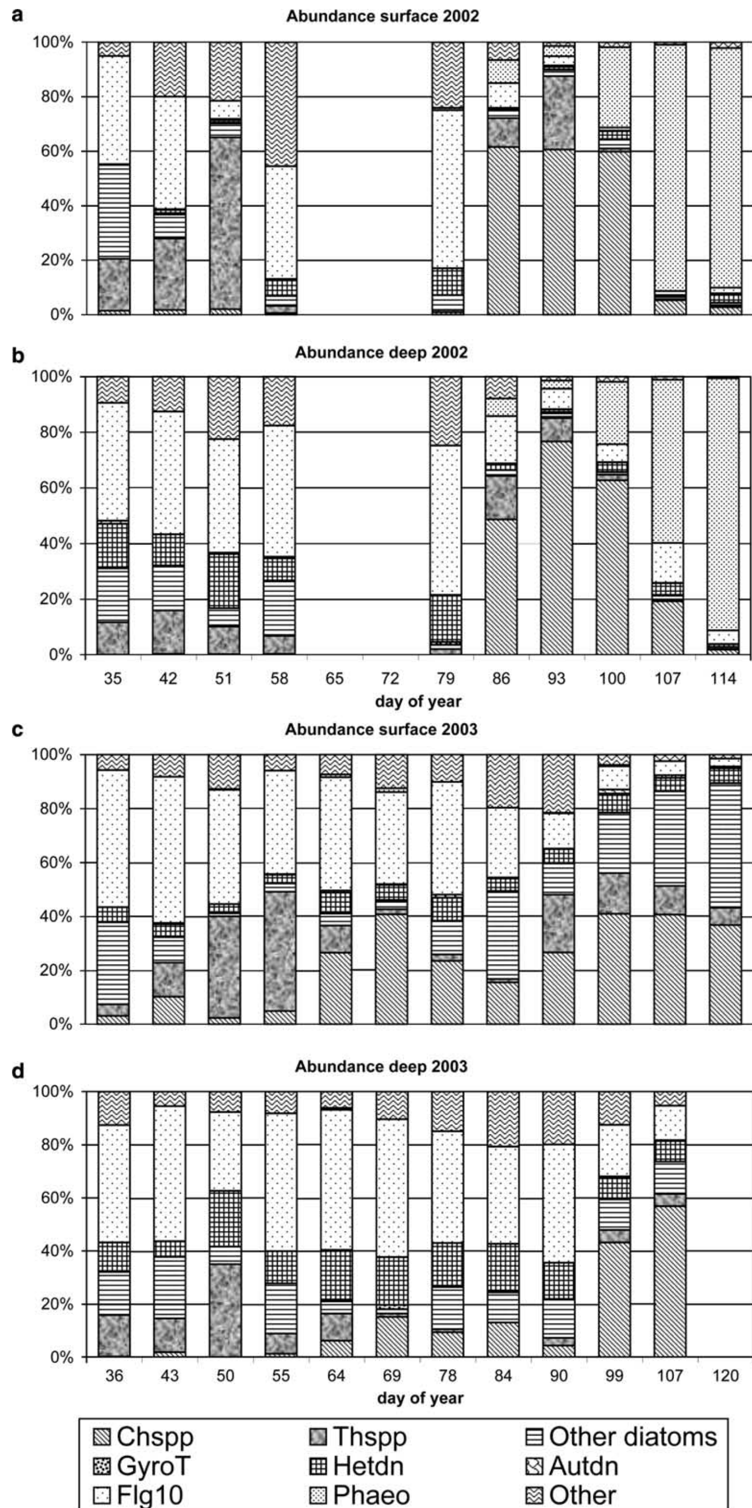


Fig. 5. Percentages of cell abundances for the nine major species or groups of phytoplankton listed in Table 3. (a) Surface 2002; (b) deep 2002; (c) surface 2003; (d) deep 2003. Chspp = *Chaetoceros* spp., Thspp = *Thalassiosira* spp., Other diatoms = all other diatoms, GyroT = *Gyrodinium* tear, Hetdn = Heterotrophic dinoflagellates, Autdn = Autotrophic dinoflagellates, Flg10 = Flagellates < 10 μm, Phaeo = *Phaeocystis,* Other = all other species.

occurred that lasted until mid-April (days 86, 93, 100), with cell numbers of $1.4 \times 10^6\ l^{-1}$. *Phaeocystis* sp., probably *P. pouchetii*, appeared in mid-March (day 79) with cell numbers increasing to $6.8 \times 10^6\ l^{-1}$ a month later on day 107. It was still present when sampling ended in late April. Colonies of both *Ch. socialis* and *P.* cf. *pouchetii* were large with many cells. Individual cells were counted in the colonies, but numbers should be considered minimal because of the size of most colonies and the difficulty in counting all cells. When colonies of either species were broken, identification of individual cells was often difficult so that *Ch. socialis* was probably counted as *Chaetoceros* spp. and *P.* cf. *pouchetii* cells as unidentified flagellates <10 μm in size.

In the subsurface layer in 2002, unidentified flagellates <10 μm were the most abundant group until well into March (day 79; Fig. 5(b)). The Other category and heterotrophic dinoflagellates were also numerous. Some *Thalassiosira* spp. cells were present. By late March (day 86), *Chaetoceros* spp., especially *Ch. socialis*, were dominant at $>5.6 \times 10^5$ cells l^{-1}, increasing to 1.2×10^6 cells l^{-1} in early April (day 93) before decreasing slightly a week later (day 100). *Phaeocystis* cf. *pouchetii,* at 7.4×10^4 cells l^{-1}, occurred starting in late March (day 86) becoming the most abundant category at 3.5×10^6 cells l^{-1} the last sampling day in late April (day 114).

In 2003, sampling again started in early February (day 36; Fig. 5(c)) with *S. costatum* at 5×10^4 cells l^{-1} as the dominant diatom species in the surface layer. It is included in the Other diatoms category in Fig. 5(c). By mid-February (days 50, 55), *Thalassiosira* spp., including *T. aestivalis*, *T. pacifica*, and *T. nordenskioeldii*, were the most abundant diatoms and remained at high densities, to 8×10^5 cells l^{-1}, until early March (day 64). In March (days 69–84), *Thalassiosira* cell numbers were low, about $10^4\ l^{-1}$, and *Chaetoceros* spp. became the dominant genus. For one week in late March (day 84), a bloom of the centric diatom *Cerataulina pelagica* occurred with cell numbers $>1.8 \times 10^5\ l^{-1}$. *Chaetoceros* spp. were still present with increasing numbers. From late March to the middle of April (days 90–107) when sampling ended, diatoms, including species of *Chaetoceros*, *Pseudo-nitzschia*, *Skeletonema*, and *Thalassiosira*, dominated the population. Flagellates < 10 μm were abundant during most cruises in 2003 with cell numbers ranging from 7×10^4 to $>3 \times 10^5\ l^{-1}$. *Phaeocystis* appeared briefly in early March (days 64–69), but as fewer than 10^4 cells l^{-1} (Fig. 5(c)).

In the subsurface layer in 2003 (Fig. 5(d)), flagellates <10 μm were the dominant group. Heterotrophic dinoflagellates were also abundant from the time sampling started (day 36) and remained relatively so until sampling ended in mid-April (day 107). *Chaetoceros* spp. were present, but were not very numerous from day 64 to day 90, increasing to 2.8×10^5 cells l^{-1} on day 107. *Thalassiosira* spp. were nearly always present ranging from about 10^3 to 2×10^4 cells l^{-1}.

Only net tows were collected for phytoplankton in 2004. In late February, *Ch. debilis* and *Thalassiosira* spp. (*T. aestivalis* and *T. pacifica*) were most abundant. *Phaeocystis* sp. occurred in late March, and *S. costatum* was present in mid-April. When sampling ended the third week of April, the centric diatom *Actinoptychus senarius*, usually found in the benthos, was the dominant diatom based on the net tows.

Other organisms that were present and sometimes abundant during the sampling periods were unidentified small flagellates, mostly <10 μm, that were enumerated by size class and may have included single *Phaeocystis* cells and a prasinophyte flagellate, possibly *Pyramimonas* sp. Heterotrophic dinoflagellates were also common, but were mostly not identified other than as heterotrophs and even that designation sometimes may be erroneous. One very large, (90–125 μm long × 70 μm wide) heterotrophic dinoflagellate, here called *Gyrodinium* "tear" because of its shape (Fig. 2), was sometimes present, but never as more than 4×10^3 cells l^{-1} (Fig. 5(a)–(d)). A group of mixed fusiform-shaped dinoflagellate cells was called *Gyrodinium* "not tear" and is included in the heterotrophic dinoflagellate group. Other heterotrophic dinoflagellates, including *Amphidinium* spp. and *Protoperidinium brevipes*, *P. conicum*, *P. depressum*, and *P. oceanicum*, were present, but most cells in this category were not identified. Autotrophic dinoflagellates, including *Scrippsiella trochoidea*, *Heterocapsa triquetra*, and *Prorocentrum minimum*, were sometimes present, but were never numerous. Cryptomonads were frequently present, sometimes in large numbers, e.g., day 58 in 2002. These were usually small, <10 μm long, but a larger, obviously heterotrophic species was also seen. Ciliates and tintinnids occurred in most samples, but were never abundant. They are included in the Other category on Fig. 5(a)–(d).

3.4. Cell carbon

Variations in phytoplankton organic carbon roughly paralleled chlorophyll (Table 5). In the upper layer in 2002 (Fig. 6(a)), *Thalassiosira* spp. comprised most of the carbon in February (days 35–51), dropping to low

Table 5
Surface and deep layer total cell abundance (cells l^{-1}), cell carbon ($\mu g\ l^{-1}$), chlorophyll concentration ($\mu g\ l^{-1}$), and carbon:chlorophyll (C:chl) ratio at the start of grazing experiments on the first day of each spring cruise in 2002 and 2003

Day (2002)	*35*	*42*	*51*	*58*	*65*	*72*	*79*	*86*	*93*	*100*	*107*	*114*
Surface abundance	176,000	502,000	1,257,000	346,000			237,000	3,140,000	2,327,000	2,377,000	7,500,000	3,795,000
Deep abundance	27,000	93,000	283,000	71,000			101,000	1,179,000	1,587,000	1,062,000	658,000	3,855,000
Surface carbon	27.7	126.4	1032.8	53.2			32.0	595.7	803.6	303.4	259.3	281.4
Deep carbon	5.0	13.3	56.2	17.6			49.8	289.6	319.6	93.5	63.1	173.7
Surface chlorophyll	1.1	1.8	12.7	0.3	0.8	0.8	0.5	18.5	20.4	10.1	5.2	5.2
Deep chlorophyll	0.2	0.2	0.6	0.1	0.4	0.1	0.2	4.4	3.1	1.9	0.6	5.2
Surface C:chl	24.2	68.5	81.2	194.0			70.2	32.1	39.3	30.1	50.1	54.2
Deep C:chl	32.7	62.5	96.7	134.6			317.0	66.2	102.0	49.3	105.0	33.4
Day (2003)	*36*	*43*	*50*	*55*	*64*	*69*	*78*	*84*	*90*	*99*	*107*	*120*
Surface abundance	237,000	475,000	535,000	609,000	822,000	507,000	269,000	625,000	1,212,000	1,047,000	1,390,000	1,713,000
Deep abundance	93,000	104,000	41,000	134,000	151,000	128,000	90,000	87,000	106,000	268,000	492,000	
Surface carbon	21.4	101.4	270.4	383.8	284.4	161.0	67.5	433.2	512.4	396.8	329.6	372.0
Deep carbon	13.3	11.3	14.1	26.6	70.8	86.2	16.0	43.4	23.6	49.8	65.4	
Surface chlorophyll	0.6	2.5	9.7	7.3	2.5	0.7	0.6	2.7	7.6	11.5	7.2	10.2
Deep chlorophyll	0.1	0.4	0.3	0.3	0.6	0.2	0.1	0.2	0.4	0.8	1.0	0.8
Surface C:chl	35.9	39.8	27.9	52.4	114.5	240.6	117.5	160.8	67.6	34.6	46.0	36.5
Deep C:chl	92.7	30.1	56.3	84.2	114.9	513.3	219.1	285.2	58.8	60.4	64.1	

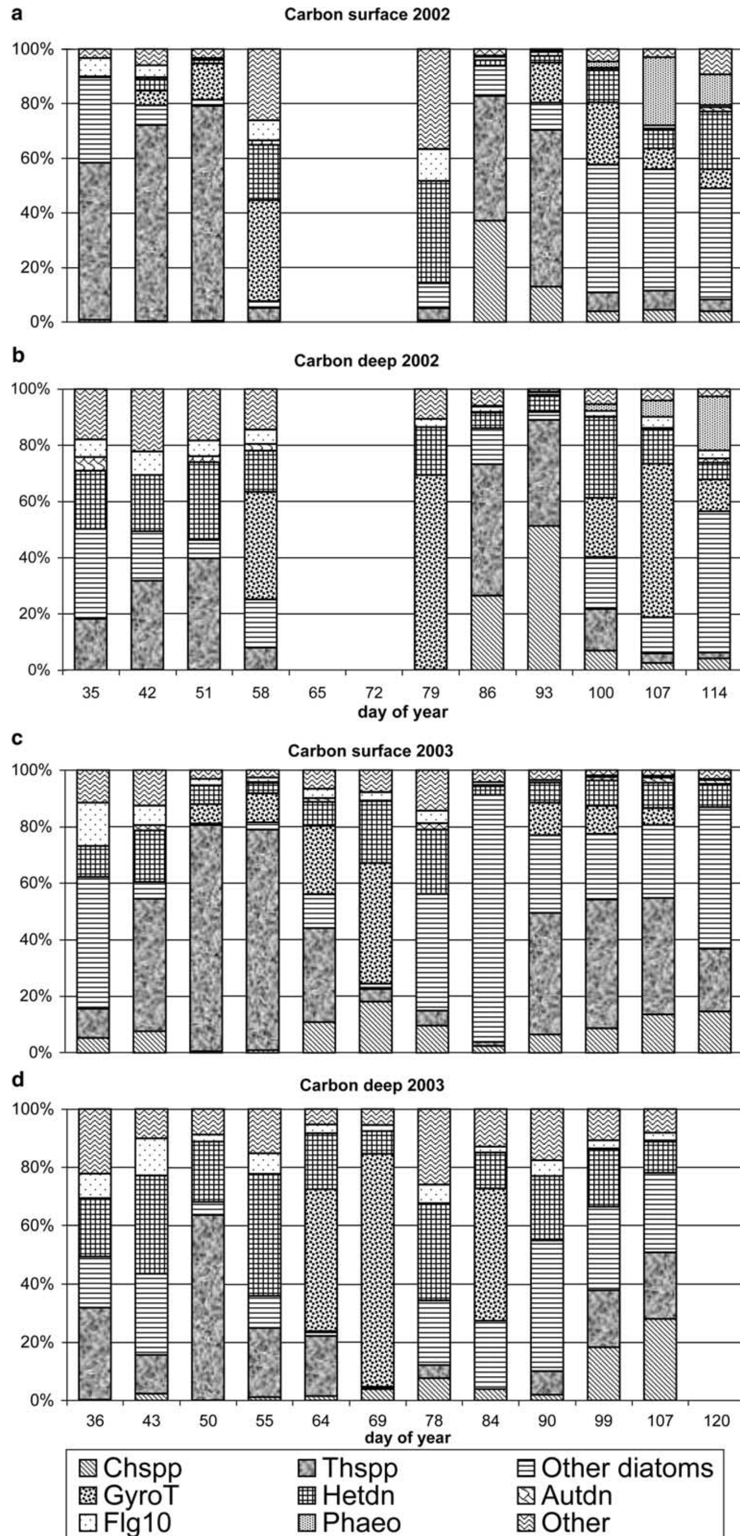


Fig. 6. Percentages of cell carbon for the nine major species or groups of phytoplankton listed in Table 3. (a) Surface 2002; (b) deep 2002; (c) surface 2003; (d) deep 2003. Key as in Fig. 5.

values in late February into March (days 58–79), then recurring at high levels in late March, early April (days 86–93) before decreasing in mid-April and remaining low until the end of the sampling period (days 100–114). Other organisms contributing to the carbon included Other diatoms on day 35 and again late in the sampling period (days 100–114) and unidentified heterotrophic dinoflagellates in late February (day 58), mid-March (day 79), and April (days 100, 107, 114). *Gyrodinium* "tear" was a major contributor to the cell carbon, especially in late February (day 58) and in April (days 93, 100, 107, 114), even though present only in small numbers. The Other category was important in late February and late March (days 58, 79) when cryptomonads were the most abundant organisms in that group.

In the deeper layer in 2002 (Fig. 6(b)), *Thalassiosira* spp. comprised most of the carbon in February (days 35–51) and again in late March to early April (days 86–93). *Chaetoceros* spp. provided 25–50% of the carbon in late March to early April (days 86, 93). Other diatoms were important early and late in the sampling period (days 35–58, 86–114), especially the last day when *Chaetoceros* spp. and *Actinoptychus senarius* were abundant. The Other category contributed to the carbon mostly in February (days 35–58), in mid- to late March (days 79, 86), and in mid- to late April (days 100–114). *Gyrodinium* "tear" was a major carbon provider in late February (day 58) and in April (days 100, 107, 114). Other heterotrophic dinoflagellates were important through much of the sampling period.

In the surface layer in 2003 (Fig. 6(c)), diatoms including *Thalassiosira* spp., *Chaetoceros* spp., and other diatoms generally contributed the most carbon. In late March (day 84) the diatom *Cerataulina pelagica* was the major contributor, although cells appeared to be unhealthy with only a few small, round chloroplasts. *Gyrodinium* "tear", present as only about 2000 cells l^{-1}, was nevertheless important during some cruises (days 50, 55, 64, 69, 90, 99, 107). Heterotrophic dinoflagellates, although mostly small in size, were often major carbon providers. Flagellates < 10 μm contributed to the carbon pool mostly during the early cruises (days 35, 43).

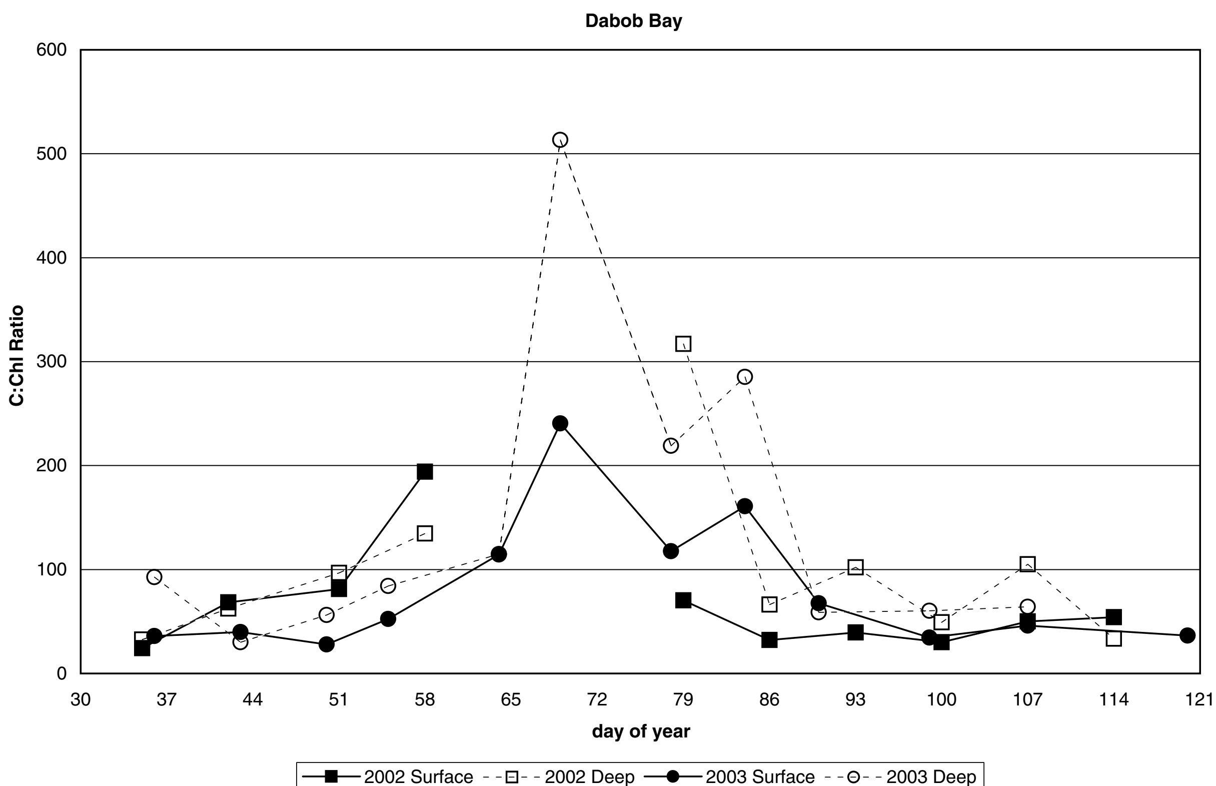
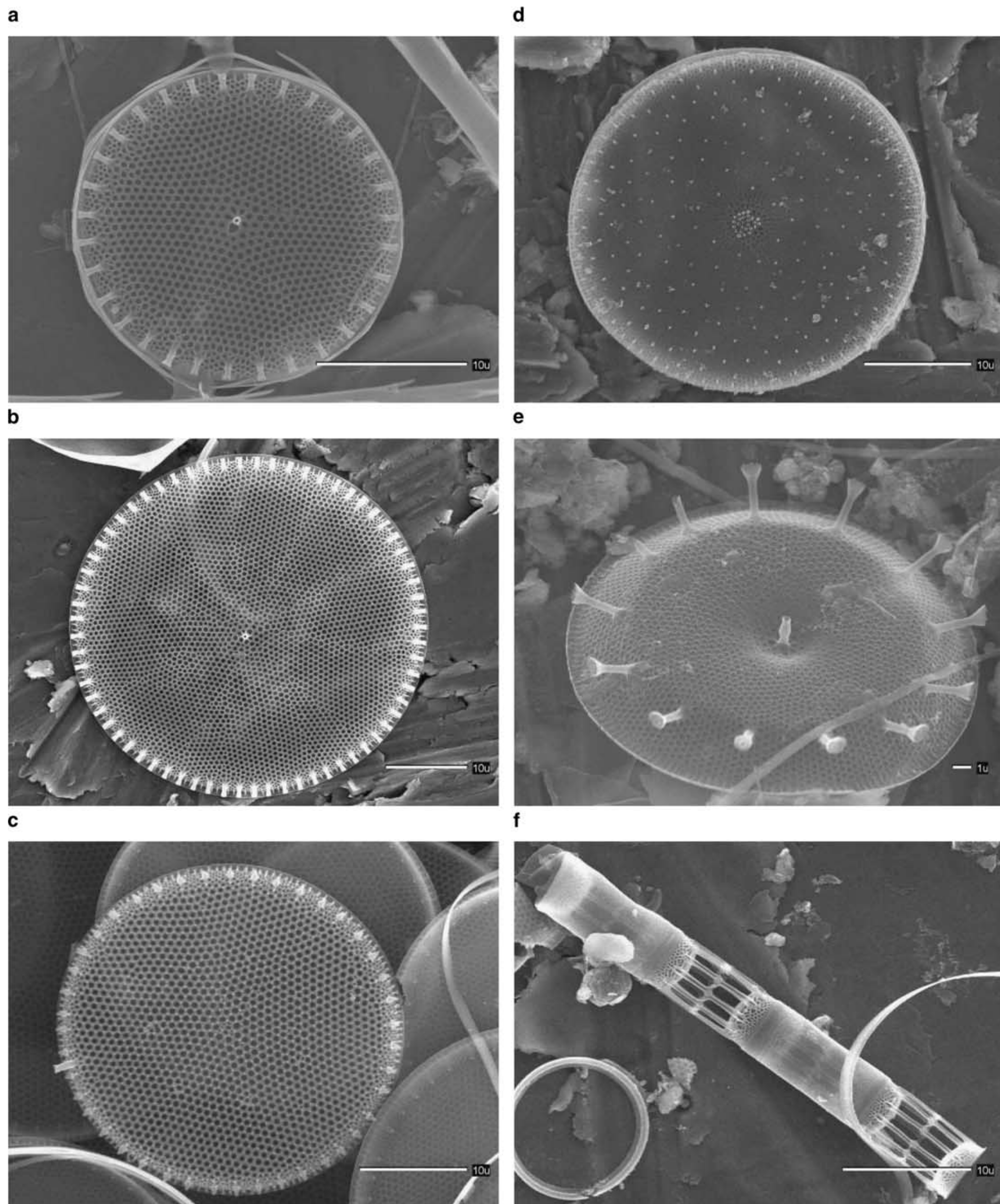


Fig. 7. Phytoplankton carbon:chlorophyll ratios for the surface and deep layers in 2002 and 2003.

In the deeper layer in 2003 (Fig. 6(d)), heterotrophic dinoflagellates were major carbon contributors through much of the sampling period. *Thalassiosira* spp. were important in February (days 36, 43, 50, 55), early March (day 64), and in mid-April (days 99, 107). *Chaetoceros* spp. were important late in the sampling period (days 99, 107). *Gyrodinium* "tear" contributed 49%, 80%, and 45%, respectively, of the carbon on the three days in March (days 64, 69, 84) when it was present even though cell numbers were <2000 l^{-1}.

The total microplankton carbon available for grazing by the mesozooplankton in the surface layer based on the maximal cell numbers observed from the initial grazing experiments ranged from 28 to over 1000 $\mu g\, l^{-1}$ in



2002 and from 21–512 μg l^{-1} in 2003. The carbon available in the deep layer was only 5–320 μg l^{-1} in 2002 and 11–86 μg l^{-1} in 2003.

The ratio of carbon to chlorophyll in the experiments was estimated from the calculated carbon based on cell counts and measurements of the initial chlorophyll in the experiment containers (Fig. 7). During both years, the ratio was lower in the February and April bloom periods than in March. In 2002, the range for the surface layer was 24–194 (median = 52, $n = 10$), with only one value >100 (day 58); the layer was generally the upper 6–12 m of water. In 2003, when the surface layer represented the upper 25 m of the water column, the range in C:chl ratio was 28–240 (median 49, $n = 12$), with four days (64, 69, 78, 84) exceeding 100.

The ratio in the deeper layer was more variable. In 2002, the range usually representing the depths between 6 or 12–25 m, was 33–320 (median = 81, $n = 10$) with four days >100 (one in February, one in March, and two in April). In 2003, the range for the 25–50 m depth interval was 30–510 (median = 84; $n = 11$) with four days in March >100. The days with the highest C:chl ratio were all days when the contributions of heterotrophic dinoflagellates and *Gyrodinium* "tear" were important. In 2002 carbon was not estimated on days 65 or 72, when the chlorophyll levels were low, and the ratio may have been high as it was in 2003.

3.5. Polyunsaturated aldehydes

Cultures of the diatoms *Ditylum brightwellii*, *S. costatum*, *T. aestivalis*, *T. anguste-lineata*, *T. nordenskioeldii*, *T. pacifica*, *T. punctigera*, and *T. rotula* and the dinoflagellate *Prorocentrum micans* were tested for PUA. Of these, *T. aestivalis*, *T. anguste-lineata*, *T. nordenskioeldii*, *T. pacifica,* and *T. rotula* tested positive for aldehyde production (Wichard et al., 2005a). Three isolates of *S. costatum* from Dabob Bay were also tested by the SPME or PFBHA-derivative approach but the cultures died before quantifications were possible. Fig. 8 shows the diatom species and Table 6 the species sorted by descending amount of released PUA and thus their proposed toxicity (Wichard et al., 2005a).

Among the PUA-producing species, *S. costatum* was the most abundant species in the surface layer when sampling began in 2002 (day 35; Fig. 9(a)), followed by *Thalassiosira* spp. Other *Thalassiosira* species were identified by day 42 and with *S. costatum* comprised 34% of the phytoplankton cells. By day 51, *Thalassiosira* spp. and *S. costatum* were >64% of the phytoplankton population with *T. pacifica* at 35% and *T. aestivalis* at 26%, the dominant species. By the end of February (day 58), aldehyde producers dropped to ~4% of the population and remained so until late March. The *Thalassiosira* spp. and *S. costatum* returned in late March (day 86)-early April (day 93) being 12% and 27%, respectively, of the population before dropping to <1% at the end of April. A similar pattern occurred in the deep layer (Fig. 9(b)) except that *S. costatum* was a slightly higher portion of the population in February. The aldehyde producers were most numerous in the deep layer at 23% on day 42 in mid-February.

The percentage composition of the PUA-producing diatoms based on cell numbers in the surface layer in 2003 (Fig. 9(c)) was similar to that in 2002 being highest in February with 46% on day 55, dropping to lower levels until the end of March (day 90), and increasing in April (days 99–107). The percentage of *S. costatum* cells was highest in early February (day 36) and mid-April (day 107) with all *Thalassiosira* species being present as higher percentages on all the other days. In the deep layer (Fig. 9(d)), the pattern was similar except that

Table 6
Phytoplankton species tested, their cell volumes, and the polyunsaturated aldehydes (PUA) they produce

Species	Cell volume (μm^3)	PUA (fmol $cell^{-1}$)	C7:2 (%)	C8:2 (%)	C8:3 (%)	C10:2 (%)	C10:3 (%)
T. pacifica	1256	9.81 ± 0.06	70	19	11	0	0[a]
T. aestivalis	4630	1.54 ± 0.26	78	16	6	0	0
T. anguste-lineata	2245	1.53 ± 0.07	69	17	14	0	0
T. rotula	16,406	1.27 ± 0.10	24	8	41	0	27
T. nordenskioeldii	3776	0.01 ± 0.00	0[a]	100	0	0	0
S. costatum	286	0.01 ± 0.00 – 0.13 ± 0.01[b]	58–81	19–38	0–3	0	0

Columns 4–8 are the aldehydes and the percent of total PUA found in each species. Aldehyde data from Wichard et al. (2005a). See Fig. 8 for SEM photos of species.

[a] Traces detected.

[b] Aldehyde data not from Dabob Bay samples; photograph in Fig. 8 from Dabob Bay samples.

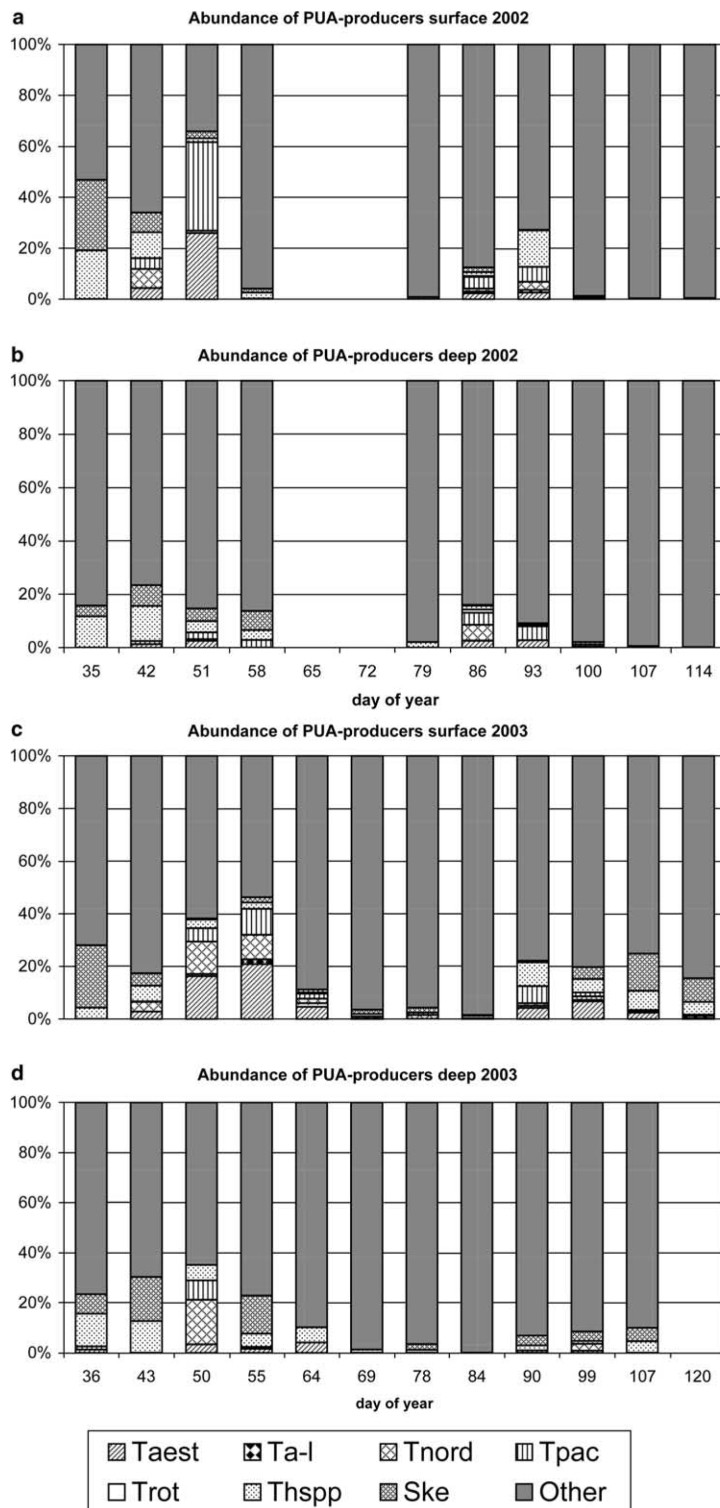


Fig. 9. PUA-producing diatoms as a percentage of total cell numbers. (a) Surface 2002; (b) deep 2002; (c) surface 2003; (d) deep 2003. Key: Taest = *Thalassiosira aestivalis*, Ta-l = *T. anguste-lineata*, Tnord = *T. nordenskioeldii*, Tpac = *T. pacifica*, Trot = *T. rotula*, Thspp = all other *Thalassiosira* species, Ske = *Skeletonema costatum*, Other = all other species.

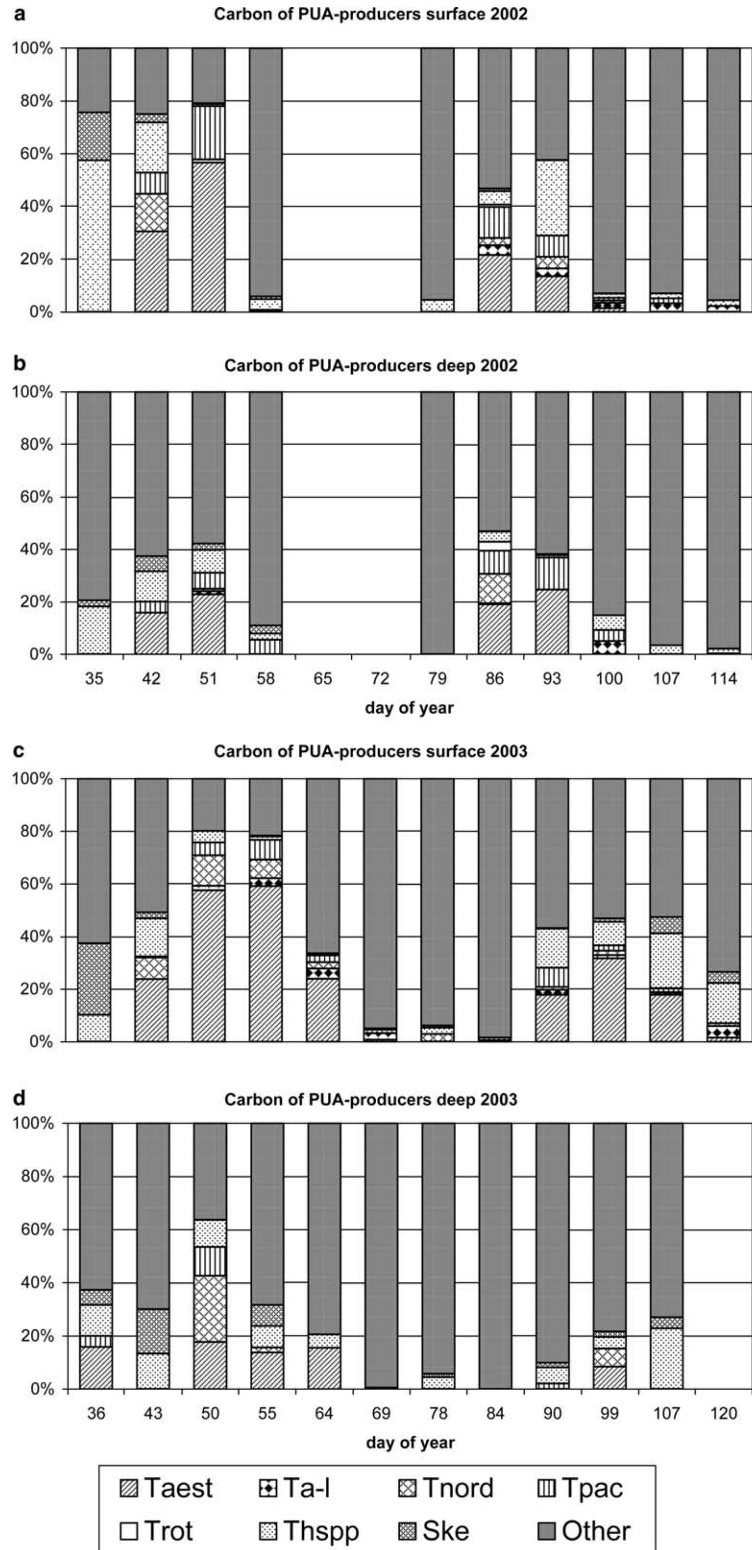


Fig. 10. Carbon in PUA-producing diatoms as a percentage of total cell carbon. (a) Surface 2002; (b) deep 2002; (c) surface 2003; (d) deep 2003. Key as in Fig. 9.

S. costatum was more numerous on days 36, 43 and 55 in February and in late March (day 90) and April (days 99, 107).

The PUA-producing diatom species, because of their relatively large size (except for *S. costatum*), were also major contributors to the phytoplankton carbon in both years (Fig. 10(a)–(d)) with highest carbon in the surface layer in February (days 35–51 in 2002, 36–64 in 2003) and again in late March to early April (days 86–93) in 2002 and April (days 90–107) in 2003. Similar patterns occurred in the deep layers.

4. Discussion

4.1. Hydrography

The late winter and springtime conditions in Dabob Bay are generally conducive to surface blooms of phytoplankton and relatively constant for the developmental stages of copepods. A layer of 3–10 m deep slightly fresher water overlies a saltier zone during the February to April period permitting intense phytoplankton blooms to develop. Solar heating warms this layer as the season progresses, while intermittent wind events maintain nutrient supply to the upper layer most of the time (Kollmeyer, 1965) and allows sustained phytoplankton growth for 1–2 week intervals.

The surface temperature range at our study site during the February-March period was 7–9 °C, and the upper 5–15 m warmed to 11–12 °C in April while the 25–50 m zone slowly warmed from about 9 to 9.5 °C between February and April. There were different patterns of temperature and salinity profiles in the two years. In 2002 temperature profiles varied a little more week to week than did the salinity profiles, while the opposite occurred in 2003. The water column was somewhat more stratified in 2003 than in 2002 or 2004.

4.2. Chlorophyll

In earlier studies (e.g., Dagg et al., 1997; Larrance, 1964; Runge, 1985; Fig. 11), phytoplankton blooms with chlorophyll levels ranging from about 150 to nearly 400 mg m^{-2} occurred in March in Dabob Bay. This was

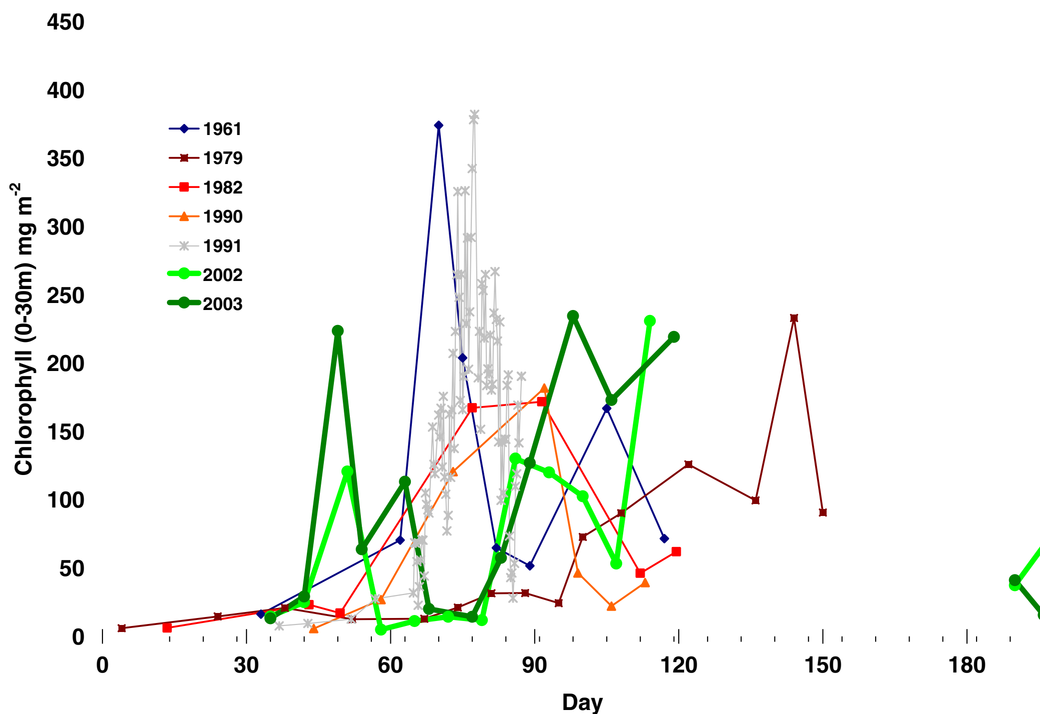


Fig. 11. Depth-integrated chlorophyll stocks in the upper 30 m in Dabob Bay (mg m^{-2}), 1961–2003.

not the case during our study when short-lived chlorophyll peaks occurred in mid-February, followed by decreases to low winter conditions lasting for one or more weeks. Second chlorophyll highs occurred in late March and into April. The timing of these blooms varied from March to May, and the duration of the blooms was sometimes less than a week or more than two weeks indicating the transitory nature of the blooms. However, local data gathered at intervals since 1961 show that multiple blooms occur every year with peak chlorophyll values ranging from 200 to 400 mg m^{-2} in the upper 30 m. The overall highest chlorophyll concentrations have been observed in March (Fig. 11).

In our samples, the chlorophyll profiles (data not shown) often displayed a subsurface peak concentration in or just above the pycnocline. Maximal concentrations were usually >20 μg l^{-1} during blooms, and <5 μg l^{-1} during non-bloom periods. Below the pycnocline, chlorophyll concentrations were usually <1 μg l^{-1} (Table 5). Thus, there were large differences in the amount of food available to the zooplankton in the two layers selected for grazing experiments each week through April, while the physical parameters (T, S) were basically the same for the two layers.

4.3. Phytoplankton species

More than 100 species or groups of phytoplankton were identified in Dabob Bay. These were diatoms, dinoflagellates, and flagellates from a number of taxonomic categories, including cryptomonads, choanoflagellates, chrysophytes (*Dinobryon* spp.), prymnesiophytes (*Phaeocystis* sp.), chlorophytes, euglenoids, silicoflagellates, and ebriids. In addition, ciliated protozoans, primarily oligotrichs and tintinnids, radiolarians, rotifers, and some invertebrate larvae were counted and/or noted in counting chambers and net tows. All of the organisms identified to species have been seen previously in Puget Sound or other Pacific Northwest waters (Horner, unpublished).

The pattern of species presence and timing seen here is similar to that found previously by Larrance (1964) and Hedges et al. (1988a) during single years and by Postel and Horner (unpublished, 1997–2001) in bi-weekly samples over three years. While some confusion may exist over specific names because of recent name changes, the organisms themselves remain the same. For example, what is called *T. decipiens* in Larrance (1964) and Hedges et al. (1988a) was likely *T. pacifica*, but was misidentified because of taxonomic confusion with *T. decipiens* and *T. angulata* (Hasle, 1979; Hasle & Syvertsen, 1996). The "unidentified, biflagellate green monad" seen by Dortch et al. (1985) may have been the same as the *Dunaliella* sp. reported by Larrance (1964). *Nitzschia seriata*, reported by Hedges et al. (1988a), has been transferred to the genus *Pseudo-nitzschia* (Hasle, 1993) and was probably *P. australis* with which it is easily confused (Hasle, Lange, & Syvertsen, 1996) and which is known to occur in Puget Sound and other Pacific Northwest waters (Horner, 2003; Horner & Postel, 1993). The *Cerataulina bergonii* bloom seen by Hedges et al. (1988a) in early April was undoubtedly *C. pelagica*. A similar bloom was seen by us in late March 2003. Thus, for over a period of at least four decades, phytoplankton species and their temporal distributions have remained more or less the same in Dabob Bay, even to blooms of individual species, e.g., *C. pelagica*, that are not often major components of the local phytoplankton community. Still other species occurred in high concentrations at irregular time intervals, e.g., *Phaeocystis* sp.

A phytoplankton bloom regularly occurs in spring in Dabob Bay, frequently in March (Fig. 11), with the bloom often dominated by *T. pacifica* (Buck & Newton, 1995; Larrance, 1964). However, during our study, the initial bloom occurred earlier, in February both years with *Thalassiosira* spp. (especially *T. aestivalis* and *T. pacifica*) as the dominant diatom species, although *S. costatum* was also abundant at the same time. Other diatoms were present, but were usually not numerous. Large numbers of small, <10 μm diameter unidentified flagellates were often present. They occurred throughout the sampling period in both years, but were more abundant in 2003. A second bloom comprised of *Thalassiosira* spp. in relatively high concentrations occurred in late March to early April both years, accompanied by *Chaetoceros* spp., including *Ch. socialis*, *Ch. debilis*, and *Ch. diadema*. In 2003, other diatoms were also present, especially *S. costatum*, *Pseudo-nitzschia* spp. and unidentified small pennate diatoms. In 2002, *Phaeocystis* cf. *pouchetii* occurred in mid-April effectively replacing the diatoms. Few *Phaeocystis* cells were seen in 2003, but it was present in high concentrations again in 2004.

In both years, although silica was plentiful, ranging from 20 to 60 μM over the course of the sampling periods, many of the diatoms, especially the *Chaetoceros* species, were weakly silicified making positive

identifications difficult or impossible. This was especially true for some of the smaller species. Also for *Chaetoceros* spp., many of the chains were broken and cells often occurred as single cells, rather than as chains thus adding to the identification problem. Even though there was still silicate in the nutrient samples, other macronutrients were occasionally measured at surface concentrations that could limit phytoplankton growth, e.g., surface nitrate and phosphate <0.5 μM occurred in April of both years (data not shown). Further, these nutrients varied enough in the upper 15 m that there were probably short intervals (<1 week) when nutrient limitation could have affected phytoplankton cell growth. Low nutrient levels did not persist in the springtime because wind events frequently mixed nutrients back into the surface layer.

Among the dinoflagellates, species of the heterotrophic genus *Protoperidinium* were usually present, but never in large numbers, possibly because all of our sampling was in spring. An apparently unnamed heterotrophic, athecate dinoflagellate identified here as *Gyrodinium* "tear" was often present, but never as more than ca. 4×10^3 cells l^{-1}. This is similar to the abundance for the same organism reported by Buck and Newton (1995) who also described its fecal pellets. In our samples it often contained large numbers of empty *T. pacifica* frustules and its fecal pellets were also often present (Fig. 2).

4.4. Carbon

The estimates of phytoplankton carbon were based on average cell size measurements from the experiments and the literature using simplified cell shapes and the equations of Menden-Deuer and Lessard (2000). For most of the dates in this study, the C:chl ratios were in the range of 25–65, well within the range (~8–200) of previously published values for healthy phytoplankton populations, leading us to believe that the values were reasonable for the purposes of this study (e.g., for Dabob Bay: Dortch et al., 1985; Welschmeyer & Lorenzen, 1985a; and elsewhere: Arin, Morán, & Estrada, 2002; Booth & Horner, 1997; Chavez, Buck, Service, Newton, & Barber, 1996). The very high values, >150, occurred in samples that had many heterotrophic dinoflagellates, including *Gyrodinium* "tear" (Buck & Newton, 1995). The value of 510 for the deeper layer on day 69 in 2003 may be higher than expected, but few pigmented cells of any kind were present and *Gyrodinium* "tear" represented over 80% of the cell carbon even though few cells were counted. However, it is possible that our calculations of carbon based on cell volume overestimated the importance of this organism.

4.5. Chemical defense and unsaturated aldehydes

Grazing-activated chemical defense is known for a number of unicellular microalgae, e.g., the coccolithophorid *Emiliania huxleyi*, where production of DMS from DMSP occurred only after the cells were ingested and lysed (Wolfe, Steinke, & Kirst, 1997). Some diatoms, including *Skeletonema costatum*, *Asterionella formosa*, and *T. rotula*, are known to release toxic compounds when cells are damaged (e.g., d'Ippolito et al., 2002; Miralto et al., 1999; Pohnert, 2000) and might provide for chemical defense. The metabolites, derived from highly unsaturated (C_{16} and C_{20}) fatty acids, are produced and released within seconds after cell breakage occurs (Pohnert, 2005). Thus, during normal growth, the cells avoid using cellular resources to produce costly secondary metabolites and also minimize the risk of self-toxicity. Yet the defensive mechanism, i.e., the production of PUA, is available when the cells are damaged by grazers (Pohnert & Boland, 2002).

The ability of different diatoms to produce PUA depends on both the species and the isolate (Pohnert et al., 2002; Wichard et al., 2005a). PUA-producing diatoms occurred during the earliest two phytoplankton blooms observed in both 2002 and 2003. In fact, a culture of *T. pacifica* isolated from Dabob Bay was the highest producer of PUA, at 9.8 fmol cell^{-1}, of more than 70 phytoplankton cultures tested (Wichard et al., 2005a). Cultures of *T. aestivalis* and *T. anguste-lineata* isolated from Dabob Bay and *T. rotula* isolated from Pt. Wells (Puget Sound near Seattle) also produced high concentrations of PUA near 1.5 fmol cell^{-1} (Table 6).

The effects of these PUA were possibly seen in our studies when *Calanus pacificus* sometimes grazed on *T. aestivalis*, but at other times avoided it (Leising et al., 2005b). Further, the reproductive success, especially naupliar survival, of both *C. pacificus* (Piersonn, Halsband-Lenk, & Leising, 2005) and *Pseudocalanus newmani* (Halsband-Lenk, Pierson, & Leising, 2005), was negatively affected following *Thalassiosira* blooms.

5. Conclusions

Multiple phytoplankton blooms, dominated by diatoms, occurred in winter and spring in Dabob Bay. Chlorophyll peaks were generally earlier (February vs. March) and later (April) than in previous studies.

The phytoplankton species seen here and their timing are similar to what have been seen previously, e.g., Dortch et al. (1985), Hedges et al. (1988a); Larrance (1964); Postel and Horner (unpublished, 1997–2001).

Gyrodinium "tear," also reported by Buck and Newton (1995), was seen to graze heavily on *T. pacifica* as evidenced by the diatom cells frequently seen inside the dinoflagellate and its sometimes large numbers of fecal pellets consisting solely of empty *T. pacifica* frustules.

Large diatoms (*Thalassiosira* spp.) were generally the major contributors of phytoplankton carbon, but small heterotrophic dinoflagellates that sometimes occurred in high numbers plus the very large *Gyrodinium* "tear" were sometimes important.

Among the dominant diatoms, *Thalassiosira* spp. and *S. costatum* were shown to produce certain PUA known to affect copepod reproduction and hatching success.

Note added in proof

The large dinoflagellate called *Gyrodinium* "tear" has been identified as *G. spirale* (Bergh) Kofoid & Swezy. See Sournia et al. (1991, p. 115, Fig. 68).

Acknowledgments

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Manuscript draft:

The influence of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes on the reproductive success of *Calanus helgolandicus* in coastal waters off Roscoff (Bretagne, France)

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Limnology and Oceanography, in preparation (2006).

Introduction:

Zooplankton species hold a central position in food webs and a combination of predation and resource limitation typically regulates the populations (Hairston *et al.* 1960, Odum 1969). The general accepted view that diatoms, as most dominant micro-phytoplankter during spring bloom, are a high quality food for zooplankton and boost secondary production was challenged by several laboratory experiments. Attention has focused on the role of the maternal diet as a factor influencing the survival of embryonic and early naupliar stages in copepod populations (Ianora & Poulet 1993, Poulet *et al.* 1994). Ban *et al.* (1997) described the varying effect of diatom-rich diets on copepod egg production rates and hatching success, whereas the feeding copepod itself is unaffected. In the last decade the work has stimulated ecologists and chemists to search for the underlying mechanism.

A negative influence of diatom-derived $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes (PUA, e.g. 2,4-octadienal or 2,4,7-decatrienal) on the reproductive success of copepods has been suggested (Miralto *et al.* 1999a, d'Ippolito *et al.* 2002a, d'Ippolito *et al.* 2002b, Pohnert & Boland 2002, Pohnert *et al.* 2002). The production of PUA is initiated upon cell disruption that would occur during mesoplankton grazing (Pohnert 2000). Wound-activated lipases such as phospholipases A_2 and galactolipases release polyunsaturated fatty acids (Pohnert 2002, d'Ippolito *et al.* 2004), which are further metabolized under the control of lipoxygenase/hydroperoxide-lyases into the reactive PUA. Since induced or activated defenses are thought to influence the flow of matter between trophic levels, a generally negative impact of diatoms on herbivores would have major implications for the traditional view of the marine food web.

Therefore numerous laboratory studies were set up in order to get a deeper insight into the diatom/copepod interaction. While some authors have illustrated a negative effect of diatom-diets on that (Miralto *et al.* 1999a, Ban *et al.* 2000, Halsband-Lenk *et al.* 2005, (**publication 5**), others have not found any adverse effect (Jonasdottir 1994, Jonasdottir *et al.* 1998, Irigoien *et al.* 2002). In these cases it is argued that poor egg production and egg viability results from nutritional inadequacies due to mono-algal diets (Schmidt & Jonasdottir 1997, Jones & Flynn 2005) rather than deleterious compounds like PUA in diatom diets. Recently a survey of the chemical defence potential of 51 diatom species has demonstrated

that only ca. 30 % of the investigated species release elevated amounts of PUA in a wide concentration range of two oders of magnitude. Some of the controversial discussions (**publication 1**) on reproductive parameters might be explained by the species and strain specific production of PUA (**publication 3**). While egg production rate seems not to be affected by PUA producing diatoms rather by an unknown factor (**publication 12**), but hatchability is affected by these metabolites (Miralto *et al.* 1999a, Pohnert *et al.* 2002).

Several studies of Ban *et al.* (1997, 2000), Starr *et al.* (1999) and Miralto *et al.* (2003) have shown that not only egg production and hatchability, but also larva development have to be examined routinely when estimating the recruitment of copepods into a planktonic population. Deformities of copepod nauplii have not yet been observed for other reasons than feeding on diatoms. Only copepods feeding artificial PUA-impregnated dinoflagellates exhibit also arrested larvae development (**publication 5**).

Up to now the knowledge about the ecological relevance of PUA is scarce, because several field studies focus on the relationship between diatom blooms and copepod reproductive success without giving any information about the actual concentration of deleterious PUA in phytoplankton (Ban *et al.* 2000, Irigoien *et al.* 2002, Devreker *et al.* 2005, Jonasdottir *et al.* 2005, Maps *et al.* 2005). Moreover, most field investigations do not provide a bias for all aspects of reproductive response (= egg production, hatching and larva development). For instance Irigorien *et al.* (2002) have rejected any negative influence on copepod hatching success in ecosystems with high diatom concentrations. However the larva survival at different copepodite stages was not measured subsequently. On the other hand Ianora *et al.* (2004, **publication 5**) and Halsband-Lenk *et al.* (2005) have correlated the occurrence of diatoms, such known as aldehyde producers, with the deformities of copepod nauplii without measuring the *in situ* PUA production of the phytoplankton.

For evaluation of the reproductive response of the calanoid copepod *Calanus helgolandicus* to the phytoplankton succession in the Western English Channel we aimed to investigate both toxic and nutritional aspects of food biochemistry. Therefore we have measured chemical (PUA, polyunsaturated fatty acids), nutritional (chlorophyll *a*, POC, PON, C/N) and abiotic parameters (temperature) in order to test the hypothesis whether PUA interfere negatively with hatching success and larva development or if there is a strong impact of nutrient limitation. The work belongs to a series of extended field experiments conducted in 2003 and 2004 in the coastal waters off Roscoff (Brittany, France). We provided an *in situ* derivatisation approach for determination and quantification of PUA in phytoplankton (**publication 2**) and studied the seasonal succession of phytoplankton and the impact of food treatments on the egg production rate of *Calanus helgolandicus* (**publication 12**).

Here we present evidence that PUA production in coastal waters of Roscoff is strongly correlated to the occurrence of the diatom *Thalassiosira rotula*. As a consequence of this

coupling elevated amounts of PUA were only determined during early *T. rotula* spring bloom. The reproductive response of *C. helgolandicus* varies during the year independent of any measured chemical and nutritional parameter. Other aspects will thus have to be taken into account in further studies.

Results:

Phytoplankton succession in coastal waters off Roscoff (Brittany, France) in 2004

The phytoplankton succession has been studied in coastal waters off Roscoff with regard to the chlorophyll concentration and the phytoplankton species since the 1970s. The chlorophyll levels near surface ranging from 0.5 µg to 7.0 µg l^{-1} and peaks in April or May, respectively. The water temperature above 1 m depth warmed from 9.95 °C in early March to 16.7 °C in September.

About 25 diatom species (proportion > 0.5 %) occurred in phytoplankton of surface near water in 2003 and 2004. The major bloom species include *Thalassiosira rotula*, *Rhizosolenia setigera* and *Guiniardia* sp. as well as *Chaetoceros* on few occasions. Whereas the genera *Chaetoceros*, *Thalassiosira* and *Rhizosolenia* are well documented in French coastal waters, *Guinardia* appears particularly in coastal waters off Roscoff during the summer. Other taxa or groups of organisms including unidentified small flaggellates < 10 µm were rare during the entire study period. The findings are similar to previous studies that describe the phytoplankton species and the succession of diatoms (Grall 1972). These results support the long-term similarities and the stability of phytoplankton species as known from other regions.

As many diatom species were present only in small numbers or on few occasions, we aimed to isolate only the dominating species that are proposed to mainly been consumed by the copepods during the phytoplankton succession (**Fig. 11**). Besides *Chaetoceros sp*. the bloom forming *T. rotula*, *R. setigera* and *G. delicatula* were isolated in spring/summer time and subsequently grown under uni-algal conditions. In addition selected other less dominant species were isolated for comparision. All isolated species were submitted to chemical analysis (PUA, total fatty acid).

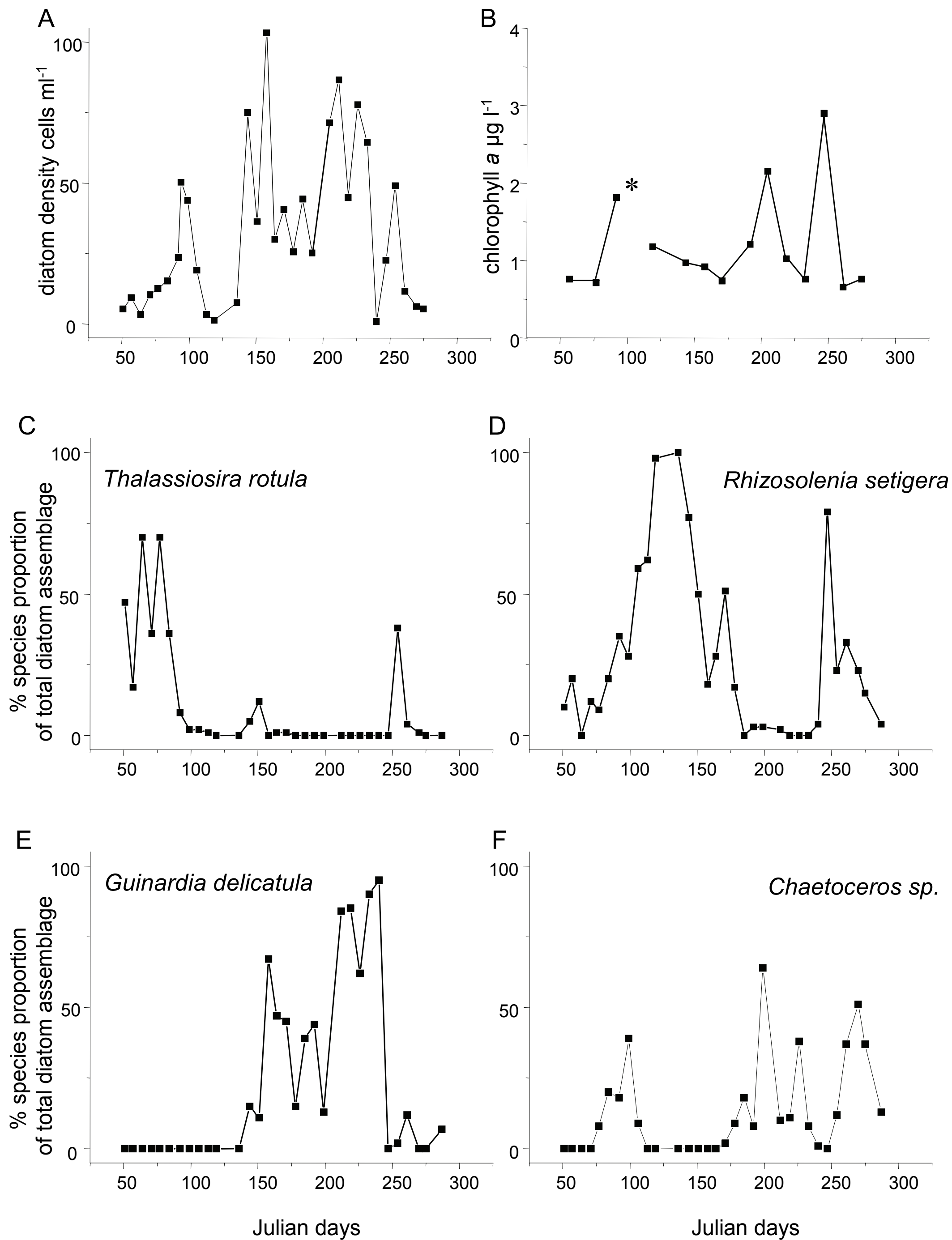

Fig. 11 Temporal change in diatom density (**A**), chlorophyll *a* concentration (1 m depth) (**B**) and in species proportion of total diatom assemblage in 2004 (**C-F**). * The main peak of chlorophyll *a* was missed in 2004. In 2003 a chlorophyll *a* peak, near to 6.5 µg l^{-1} occurred in this period.

Effects of nutritional parameters and water temperature

Neither egg production nor hatching success nor any measured nutritional parameters (chlorophyll *a*, diatom density, POC, PON) were correlated during the entire study period from March to October (**Table 1**). Only the water temperature (1 m depth) and the egg production correlated slightly.

The absence of correlation between reproductive response and any environmental or food factor inquire for the food quality of the phytoplankton during the study period. Therefore the C/N ratio, an index of food quality (**Table 1**), and the amount of polyunsaturated fatty acids, particularly EPA and DHA, were measured and correlated with reproductive response factors (**Table 2**). Again these parameters did not correlate neither with egg production rate nor hatching success. Anyway, the occurrence of *T. rotula* in phytoplankton correlates with the egg production rate indicating the favourable influence of this species on the reproductive response. Moreover, the correlation between egg production rate and hatching success supports previous findings (**Table 2**) that high egg production rates tend to result in high quality eggs that hatch, while clutches of eggs spawned at low rate can be of reduced viability (**Fig. 12**). If the overall proportion of spawning females ($\boldsymbol{E}_r > 0$) is reduced, these females will produce few eggs of reduced viability (**Fig. 12**).

Diatom-derived unsaturated aldehydes in natural diatom assemblages

In situ field measurements of PUA production and reproductive response of *C. helgolandicus* were conducted simultaneously. As the Pearson correlation coefficients have suggested only *T. rotula* releases elevated amounts of PUA (**Table 2**), Accordingly the production of PUA and occurrence of *T. rotula* in phytoplankton fits perfectly the linear regression ($r^2 = 0.962$, $p < 0.001$, number of x values = 25, **Fig. 13**/insert). Because of that strong correlation, *T. rotula* might be used as a PUA-biomarker in costal waters off Roscoff.

However, the composition of produced unsaturated aldehydes changed during the season. Spring bloom provides elevated amounts of heptadienal, octadienal and decatrienal contributing nearly 95 % to the determined total amount of PUA, while the summer bloom species contribute mainly or even exclusive of heptadienal (**Fig. 13 C**). These findings correspond to the pattern of unsaturated aldehydes produced by the isolated key species *T. rotula* (spring bloom) and *G. delicatula* (summer bloom) (**Publikation 3**). In fall *T. rotula* proposed to be responsible for PUA production did not release any decatrienal and might be a different genus than in the spring bloom.

Positive correlation has also been found between total PUA and the fatty acids EPA and 16:3, which are the major precursor of these PUA.

	Chl *a* µg l^{-1}	c[diatom] number ml^{-1}	POC	PON	C/N	Water-Temp.
E_r	0.107	0.469	-0.255	-0.246	0.163	-0.605 *
H%	-0.228	0.388	-0.206	-0.246	-0.257	-0.231

Table 1 *Calanus helgolandicus*. Spearman correlation coefficients between reproductive response factors (egg production rate $\boldsymbol{E_r}$, hatching success ***H%***) and evironmental factors measured during the entire study period from March to October in 2004. Significance of correlation is given by asterisk: * $P < 0.05$; n = 14

	PUA	E_r	*H%*	14:0	16:3	16:4	20:5	22:6	DHA/ EPA	16:1/ 16:0	TRR	RS	GD	Ch
PUA		**0.467**	**-0.224**	**0.511** *	**0.571** **	**0.278**	**0.467** *	**0.399**	**-0.530** *	**0.129**	**0.762** ***	**0.301**	**-0.244**	**0.172**
E_r	**0.467**		**0.563** **	**0.399**	**0.273**	**0.226**	**0.455**	**0.393**	**-0.161**	**0.088**	**0.647** **	**0.542**	**0.077**	**-0.124**
H%	**-0.244**	**0.563** **		**0.062**	**-0.062**	**0.217**	**0.091**	**0.003**	**0.017**	**0.247**	**0.028**	**0.307**	**0.480**	**-0.083**

Table 2 *Calanus helgolandicus*. Pearson correlation coefficients between polyunsaturated aldehydes **PUA**, egg production rate $\boldsymbol{E_r}$, hatching success ***H%*** and major fatty acids determined in phytoplankton, the ratio of DHA/EPA and 16:1/16:0 as well as the four most dominant diatom species during the entire study period from March to October in 2004. **TRR** = *Thalassiosira rotula*, **RS** = *Rhizosolenia setigera*, **GD** = *Guinardia delicatula*, Ch = *Chaetoceros* sp. Significance of correlation is given by asteriks: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ n = 18

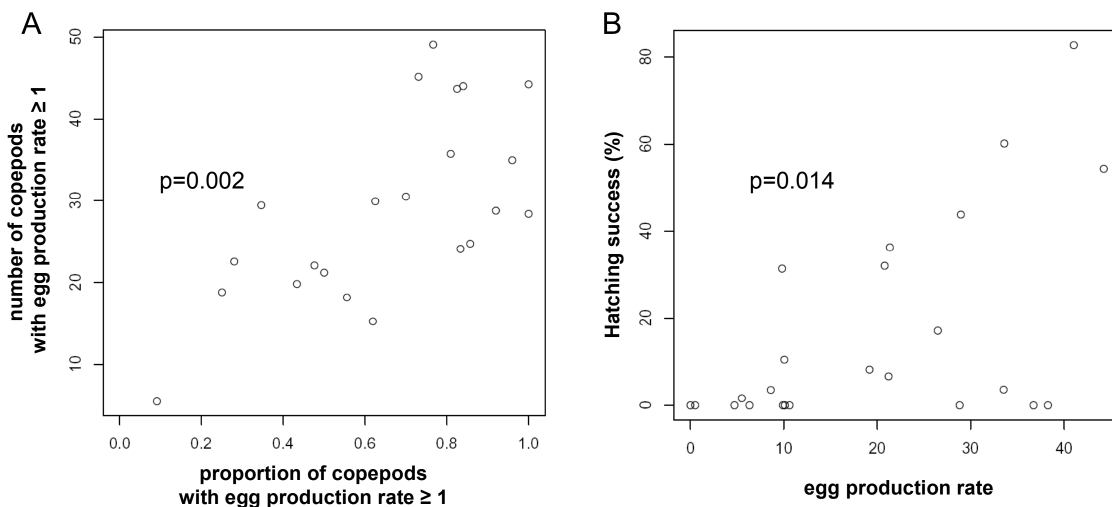

Fig. 12 *Calanus helgolandicus*. **(A)** Egg production rate of spawning copepods as function of the proportion of spawning copepods. **(B)** Hatching success ***H%*** as a function of egg production rate.

Independent of the amount of produced PUA the hatching success of *C. helgolandicus* varies in the range from 0% to 80% during the phytoplankton succession. According to tendency, hatching success is increased in early summer (May-July) compared to the spring (March-April) and significantly higher than in late summer and fall (August-October) (Wilcoxon´s test; $p < 0.01$) (**Fig. 13**)

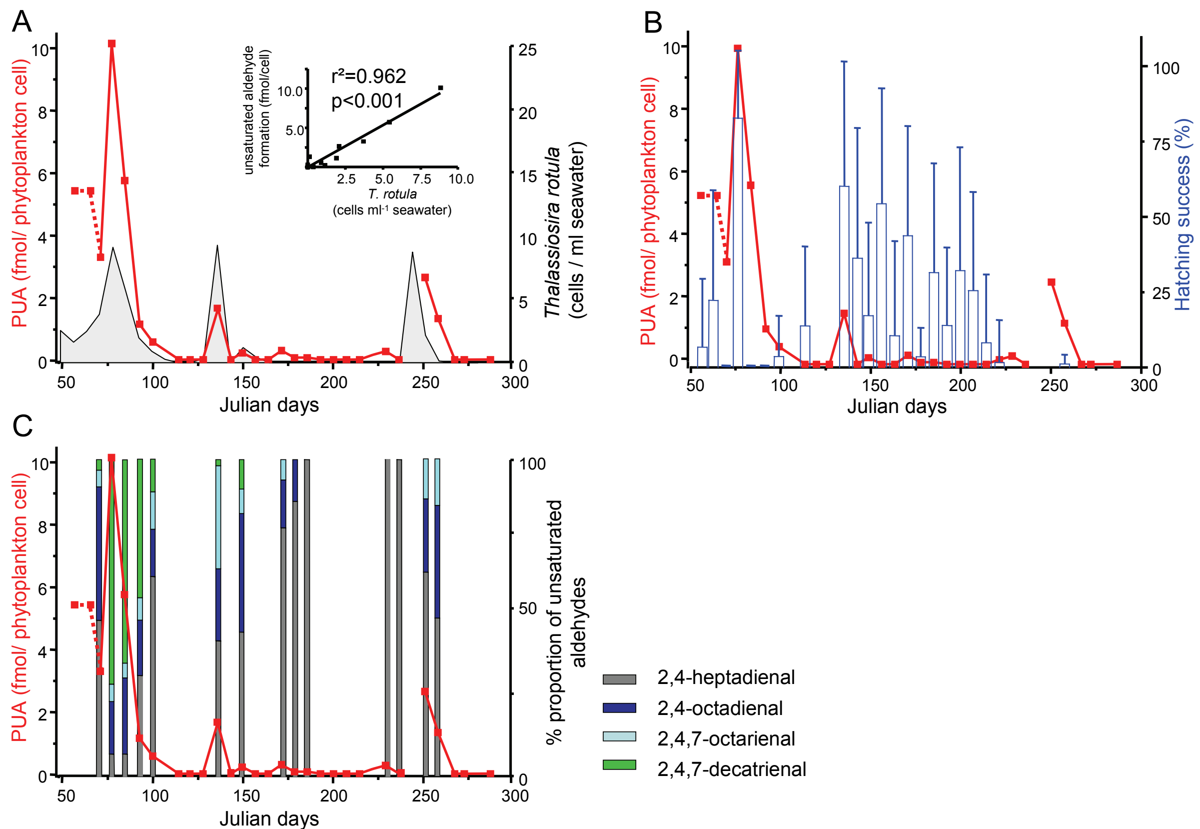

Fig. 13 Temporal changes in PUA production of natural diatom assemblages and hatching success of *C. helgolandicus* in the Western English channel (**A**). Amount of unsaturated aldehydes (red line) released by natural diatom assemblages correlates with the occurrence of *T. rotula* (light grey area). (**B**) Variable hatching success (blue bars) of *C. helgolandicus* varies in the range from 0% to 80% during the phytoplankton succession. Error based on mean + SD (n = 18). (**C**) The composition of PUA changed during the season. Dashed red line indicating approximative PUA production without quantification due to failure of quantification.

To verify if the variable hatchability is partly due to the age of the copepod-females, the beneficial non-diatom diet *Prorocentrum minimum* (PM) was applied as control food. The diet was altered between NDA (= natural enriched phytoplankton; cell size > 10 µm) and PM. After the shift of the diets the egg productive rate along with the hatching success changes dramatically (**Fig. 14**). Therefore both egg production and hatchability were reversible, following a 2 to 3 d period. This finding supports again that viable eggs tend to hatch and that there is no age effect on copepod females.

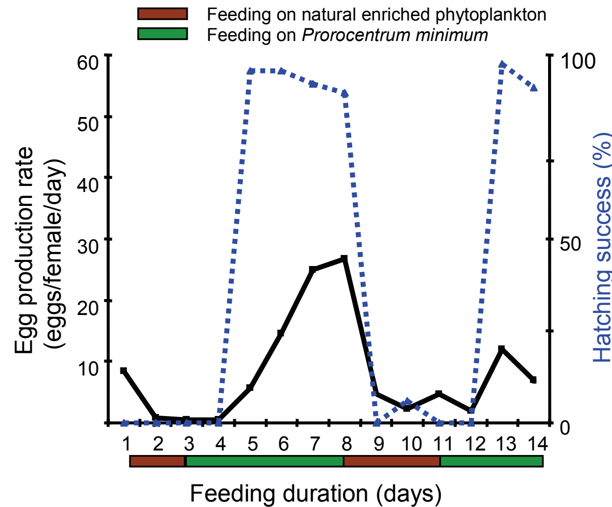

Fig. 14 *Calanus helgolandicus*. Representative control experiment of mean daily egg production (line) and hatching success (dashed line) with alternating NDA and PM diets. Mean value on Day 1 represents estimated rates in filtered seawater, reflecting E_r and *H%* in the field.

Feeding experiments with diatom diets

In order to evaluate the findings of the field study *C. helgolandicus* was fed with the freshly isolated strains *T. rotula* and *G. delicatula* that release elevated (5.7 fmol PUA cell^{-1}) or minor amounts (0.18 fmol PUA cell^{-1}) of PUA. **Fig. 15** shows the reproductive response of *Calanus helgolandicus* on these monospesific diets. The hatching success remained constantly high during the feeding on *T. rotula*. There was only a weak significant difference on day 8 compared to the initial value on day 1. Because of the high egg viability, the early stages of larval development could be examined. *T. rotula* did not affect significantly the larval development despite a slight increase of deformities. Moreover, with *G. delicatula* diet on reproductive response recovered first but continuous feeding reduced hatching success steadily and larvae deformitities increased. Hence our results suggest that hatching success and larva abnormalities in the field as well as in laboratory experiments was independent of the production of diatom-derived PUA.

With a diet of the isolate *Rhizosolenia setigera* (0 fmol PUA cell^{-1}) the egg production rate drops sharply down as previous reported (**publication 12**). Therefore further investigations on the reproductive response were not possible.

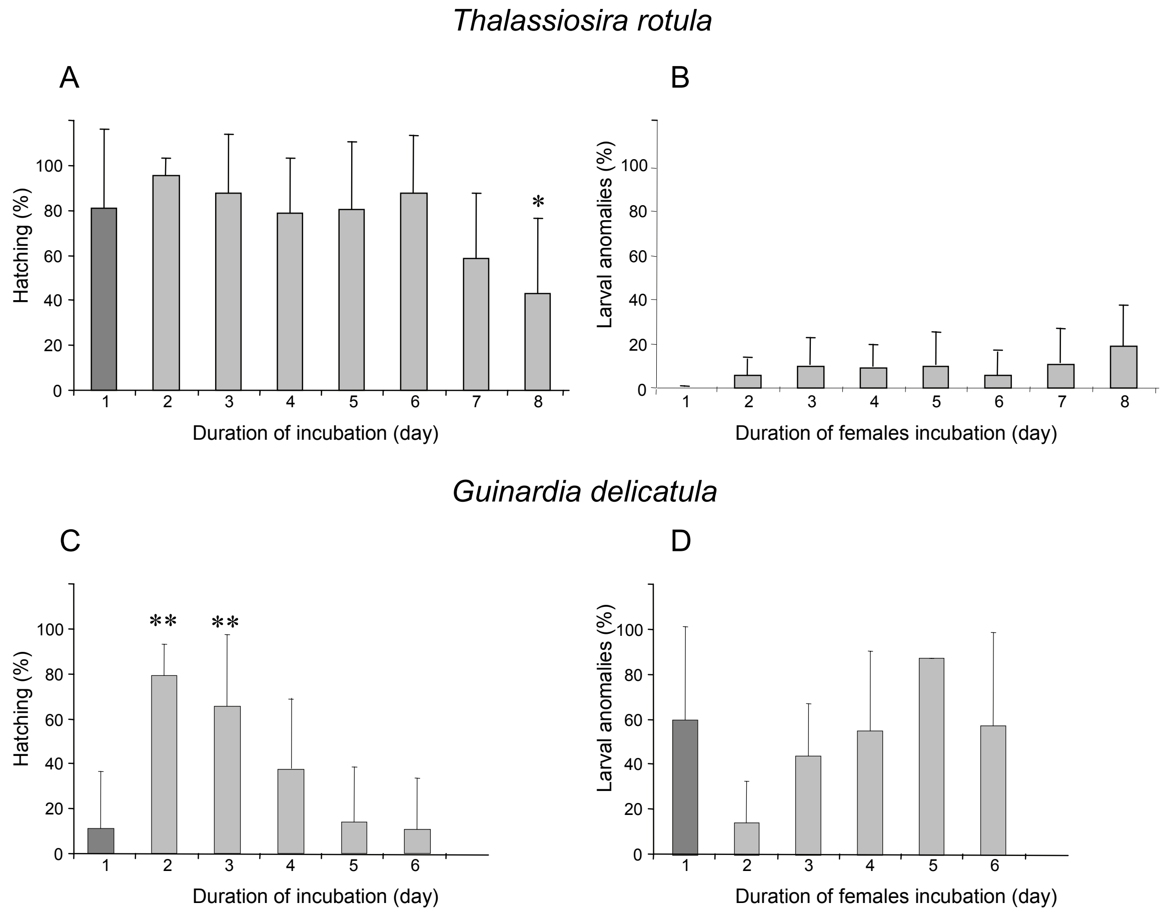

Fig. 15 Effects of diet on offspring fitness. Differences of *H* % between initial values on Day 1 (dark grey) and those on following days (light grey) with experimental diets were tested with the nonparametric Wilcoxon signed-rank test (significance level: * *P* < 0,05). Error based on mean + SD (n = 18). *Thalassiosira rotula*: (**A**) After eight days of feeding, the viability of eggs spawned by *C. helgolandicus* fed to *T. rotula* decreased slightly to 50 %. (**B**) The larval development was not affected by the diet. *Guinardia delicatula*: (**C**) The hatchability increased to 80 % first and was then reduced steadily to the baseline. (**D**) The larvae development reflects the hatchability. Because of reduced hatching success, statistical interpretation of that sampling range was not feasible.

Feeding experiments with Bis-BODIPY FL C_{11}-PC *impregnated Diatoms*

In order to prove the wound acitvated *in vivo*-production of PUA under the control of a phospholipase A_2/lipoxygenase/hydroperoxide lyase activity, the molecular probe *Bis*-BODIPY FL *C_{11}-PC* [9] was used for detection of phospholipase A_2. *C. helgolandicus* was fed on the diatom *Stephanopyxsis turris* that was impregnated with the intramolecularly quenched BODIPY-labeled phospholipid analog (demonstrated by the addition of commercial available phospholipase) (**Fig. 16 B**). Such a diet allows the continuous monitoring of the phospholipase A action in the gut under the confocal microscope with argon-ion laser excitation source, because only the separation of one of the acyl chains by a phospholipases activity results in increased fluorescence. Indeed high fluorescence was detected in the fore gut compared to other parts of the gut implying the fore gut is the site of cell disruption and activation of the diatom-enzyme cascade (**Fig. 16 C**).

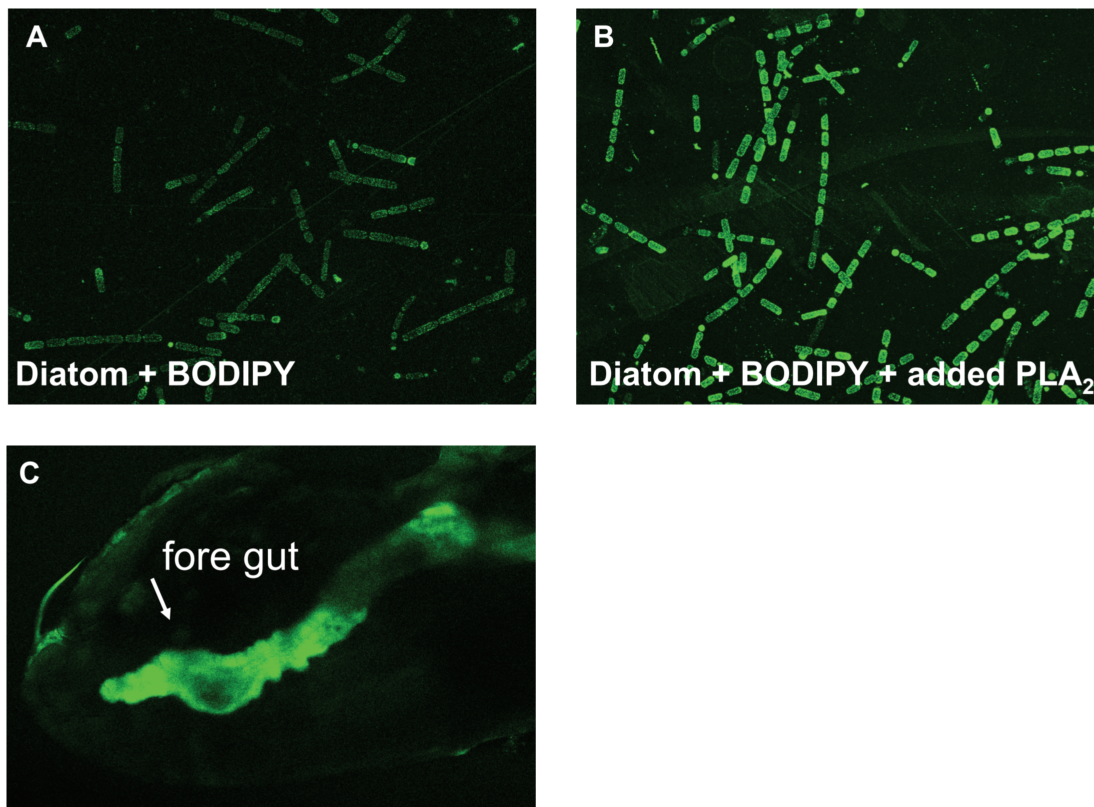

Fig. 16 Detection of phospholipase activity in the gut of *C. helgolandicus* under the confocal microscope. *S. turris* incubated with BODIPY (**A**) and subsequently treated with buffered PLA_2 (**B**) or fed to *C. helgolandicus* (**C**). Fluorescence was emitted by excitation (argon-ion laser) at 488 nm.

[9] ***Bis*-BODIPY FL *C_{11}-PC***: 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine

4 Diskussion

Ausgangspunkt der vorliegenden Arbeit war die Beobachtung, dass eine diatomeenreiche Ernährung den Reproduktionserfolg von Kopepoden negativ beeinflusst. $\alpha,\beta,\gamma,\delta$-ungesättigte Aldehyde (PUA) wurden als Verteidigungsmetaboliten identifiziert, die die Diatomeen-Kopepoden Interaktionen beeinflussen (Miralto *et al.* 1999a, Pohnert 2000). Die mögliche ökologische Bedeutung der PUA wurde durch die Formulierung einer aktivierten chemischen Verteidigung von Diatomeen (Pohnert 2000) sowie durch die Ausweitung dieses Konzepts auf benthische marine Ökosysteme (Caldwell *et al.* 2002, Caldwell *et al.* 2003) verdeutlicht. Eine Vielzahl mariner biologischer Testsysteme wurde entwickelt, um die biologische Aktivität der PUA zu belegen (Romano *et al.* 2003, Tosti *et al.* 2003), ein Struktur-Wirkungungs-Prinzip (Adolph *et al.* 2003) zu finden oder den potenziellen Einfluss auf andere marine Organismen wie Austern oder Seeigel zu verstehen (**Publikation 6**).

Die Wechselwirkungen zwischen Phyto- und Zooplankton sind aber nur dann zu verstehen, wenn möglichst viele Parameter simultan mit hoher zeitlicher Auflösung gemessen werden (Lampert & Schober 1978). Unter diesem Aspekt wurden Freilandversuche geplant, die das Ziel hatten, die Ursachen der Reduktion des Fortpflanzungserfolges unter besonderer Berücksichtigung des Einflusses von PUA zu erkennen. Die Lipoxygenase-vermittelten Biosynthesen von Oxylipinen wurden darüber hinaus an Diatomeen und dem Modellorganismus *Physcomitrella patens* untersucht.

4.1 Quantifizierung und Stabilisierung der Stereochemie ungesättigter Aldehyde

Zahlreiche Methoden sind entwickelt worden, die das Reagenz *O*-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamin-hydrochlorid (PFBHA·HCl) einsetzten, um z.B. gesättigte Aldehyde und Prostaglandine in biologischen Matrices zu bestimmen (Luo *et al.* 1995, Weisser *et al.* 1997, Watzer *et al.* 2002). Da PFBHA auch in wässrigen Lösungen mit Aldehyden und Ketonen reagiert (Spiteller *et al.* 1999) und nicht mit der Biosynthese von PUA interferiert (**Publikation 2**), eignet sich diese Derivatisierung hervorragend zum Nachweis der verwundungsaktivierten Oxylipinbildung im Phytoplankton. In der Tat erhöht die Zugabe des Reagenz zur Diatomeenkultur, gepuffert bei einem physiologischen pH-Wert, die Genauigkeit der Analytik. Die nach Wundaktivierung produzierten $\alpha,\beta,\gamma,\delta$-ungesättigten Aldehyde werden stabilisiert und die Stereochemie fixiert. Vergleichende Untersuchungen haben gezeigt, dass die spätere Zugabe des Derivatisierungsreagenz zu verwundeten Diatomeen zu geringeren Wiederfinderaten der reaktiven Metabolite führt (**Publikation 2**).

Die Bestimmung $\alpha,\beta,\gamma,\delta$-ungesättigter Aldehyde in der exponentiellen Wachstumsphase einer *Skeletonema costatum* (CCMP 2092)[10,11] Kultur unter Verwendung des PFBHA- bzw. eines Wittigsalz basierenden (d'Ippolito *et al.* 2002) Derivatisierungsreagenz ergaben einen vergleichbaren PUA-Gesamtgehalt verschiedener Kulturen des gleichen Isolats (PFBHA-Reagenz-Methode: 161 fg Zelle^{-1}, SEM = ± 20 fg; Wittig-Reagenz-Methode: 132 fg Zelle^{-1}, SEM = ± 40 fg: persönliche Mitteilung von Dr. Raffaella Cassotti).

Die Nachweisgrenze der auf PFBHA·HCl basierten Derivatisierung ist bei GC/MS Detektion (Elektronenstoßverfahren; EI-MS) im Vergleich zu bekannten Methoden wie der Mikrofestphasenextraktion (Maier *et al.* 1996) und der auf einer Reaktion mit dem Wittig-Reagenz Carboethoxyethyliden beruhenden Derivatisierung (d'Ippolito *et al.* 2002b) um rund zwei Größenordnungen verbessert worden. Durch Verwendung der chemischen Ionisierung (CI-MS) anstelle des Elektronenstoßverfahrens wird die Empfindlichkeit der Detektion von pentafluorierten Oximderivaten um weitere zwei Größenordnungen erhöht, so dass die Nachweisgrenze bei 4,4 pg PUA pro Injektion liegt. Unter der Annahme, dass eine Diatomeenzelle z.B. 0,1 fmol Octadienal produziert (*Guinardia delicatula*; Fundort: Roscoff), reichen 500 Zellen (= 6,2 pg: CI-MS) bzw. 50000 Zellen (= 0,62 ng: EI-MS) für den Nachweis von Ocatdienal aus. Während des Jahreszyklus des Phytoplanktons variierte die Zelldichte der Diatomeen von $1,3*10^3$ Zellen bis $1,0*10^5$ Zellen l^{-1}, wobei Konzentrationen unter 10^4 Zellen l^{-1} in den Küstengewässern vor Roscoff die Ausnahme sind (**Publikation 14**). In anderen Gebieten der französischen Küste werden Zellkonzentrationen von 10^4-10^6 Zellen l^{-1} gefunden (Gailhard *et al.* 2002). Eine Probennahme von 30 l Meerwasser ist daher für die routinemäßige PUA-Analytik hinreichend (**Publikation 2**).

Die PFBHA-Derivatisierung hat sich als eine einfache, schnelle und robuste *in situ*-Methode zur Bestimmung und Quantifizierung von PUA herausgestellt (**Publikationen 2, 3**), die mittlerweile von verschiedenen marinen biologischen Stationen begleitend zu Fütterungsexperimenten und anderen Fragestellungen eingesetzt wird (**Publikation 1,** Dr. Gary Caldwell pers. Mitteilung). Die *in situ*-PFBHA-Derivatisierung emöglicht darüber hinaus das Abfangen von Intermediaten, die enzymatisch isomerisiert oder metabolisiert werden. Insbesondere die Lipoxygenase vermittelte Biosynthese von (2*E*)-Nonenal und der 11-oxoundeca-5*Z*,9*E*-diensäure durch *Physcomitrella. patens* wurde durch das Abfangen der intermediären *Z*-Isomeren, (3*Z*)-Nonenal und 11-oxoundeca-5*Z*,8*Z*-diensäure, aufgeklärt. Deshalb kann man eine (3*Z*)-(2*E*)-Isomerase postulieren, die das eigentliche Endprodukt, (2*E*)-Nonenal, der Biosynthese bildet, das aufgrund seines Michael-Akzeptor-Strukturelementes einen reaktiveren Metaboliten darstellt (**Publikation 10**). Die schnelle

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[10] Sarno et *al*. (2005) haben vorgeschlagen dieses Isolat in *Skeletonema marinoi* SARNO ET ZINGONE, sp. nov. umzubenennen.

[11] **CCMP** = Center of Culture of Marine Phytoplankton Main, USA

kontrollierte (3*Z*)-(2*E*)-Isomerierung eröffnet dem Organismus somit eine Möglichkeit der Modulation biologischer Aktivität von Metaboliten (Phillips *et al.* 1979, Noordermeer *et al.* 1999, **Publikation 10**).

4.2 Reihenuntersuchung von 51 Arten auf α,β,γ,δ-ungesättige Aldehyde

Die PFBHA Derivatisierung wurde in einer Reihenuntersuchung von über 70 Diatomeenisolaten (= 51 verschiedene Arten) eingesetzt, die teilweise begleitend zu Freilandversuchen in den Gewässern von Dabob Bay und Roscoff isoliert worden sind (**Publikationen 12-14**). 38 % der untersuchten Isolate produzieren nach Zellverletzung PUA im Bereich von 0,01 - 9,8 fmol Zelle^{-1} (**Publikation 3**) und zwei weitere Arten die weniger aktive 12-Oxododeca-5*Z*,8*Z*,10*E*-triensäure (= 12-ODTE). Bemerkenswert ist auch die hohe Variabilität der PUA-Produktion von 0,15-6,34 fmol Zelle^{-1} innerhalb einer Art (*Thalassiosira rotula*).

Die Normalisierung der Aldehydproduktion auf den Chlorophyll- oder Kohlenstoffgehalt resultiert in einer höheren Bewertung einzelner sehr kleiner Arten wie *S. costatum* und *S. pseudocastum* innerhalb der Gruppe der PUA-Produzenten, doch bleiben Vertreter der *Thalassiosira* Spezies auch bei dieser Auswertung die stärksten Produzenten (**Publikation 3**), so dass die hohe Varianz in der PUA-Produktion nicht auf den unterschiedlichen Zellvolumina beruht.

Interessanterweise sind die stärksten Produzenten von PUA auch die Arten, die die Blüten in der Dabob Bay und Roscoff im Frühjahr ausbilden (**Publikationen 13, 14**). Dass blütenbildende Arten zu den stärksten Produzenten gehören, wird zudem durch die Isolate *S. costatum* (CCMP 2092) oder *T. rotula* (CCMP 1647) aus der Nord-Adria belegt. Diese Isolate produzieren im Vergleich zu anderen Fundstellen der gleichen Art bis zu zwei Größenordnungen mehr PUA pro Zelle.

Die untersuchten Isolate lassen sich hinsichtlich ihres Produktspektrums in folgende Gruppen einteilen:

I. PUA-Produzenten (z.B.: *Thalassiosira rotula*) : **14 Arten**
II. Produzenten einer Oxosäuren und einem Kohlenwasserstoff (z.B.: *Asterionella formosa*): **zwei Arten**
III. Produzenten einer Oxosäure und eines halogenierten Kohlenwasserstoff (*Stephanopyxis turris*): **eine Art**
IV. keine Produktion von PUA („Nicht-Produzenten“; z.B.: *Thalassiosira pseudonana*): **34 Arten**
V. Kontrollgruppe (Dinophyceae und Prymnesiophyceae): keine Produktion von PUA (z.B.: *Prorocentrum minimum*).

Bei Freiland- und Laborversuchen mit dem Ziel den Einfluss der PUA auf die Kopepodenentwicklung zu bewerten, erfordert die hohe innerartliche Varianz der PUA-Produktion eine begleitende chemische Analytik. Möglicherweise können ungesättigte Aldehyde als taxonomisches Unterscheidungsmerkmal einzelner Arten der Gattung Thalassiosira herangezogen werden, wie es für die VOC[12] bei den Cyanobakterien *Anabaena* gezeigt worden ist (Tasch 1985).

Des Weiteren stellt sich die Frage nach den Ursachen der hohen Variabilität. Unterschiedliche Enzymaktivitäten und Lokalisierungen der Enzyme sowie ihrer Substrate könnten die Produktspektren beeinflussen (d'Ippolito *et al.* 2004, d'Ippolito *et al.* 2006). Erste Experimente haben bereits gezeigt, dass Polyenfettsäuren, die für die Biosynthese der Aldehyde relevant sind, von PUA-Produzenten (*T. rotula*) innerhalb der ersten Minute nach Verletzung nahezu vollständig umgesetzt werden. *T. pseudonana* dagegen zeigt keine Fettsäure transformierenden Enzymaktivitäten infolge einer Zellverletzung. Die Bacillariophyceae sind hinsichtlich ihrer qualitativen Fettsäurezusammensetzung sehr einheitlich (Dunstan *et al.* 1994). Neben einem typischen 16:1/16:0 Verhältnis und EPA enthalten alle Diatomeen die vielfach ungesättigten C16-Fettsäuren 16:3 n-4, 16:4 n-1. Interessanterweise setzten einige Isolate wie *T. rotula* (CCMP 1647) sowohl Eicosapentaensäure (= EPA) als auch C16-Polyenfettsäuren zu Decatrienal und Heptadienal bzw. Octadienal und Octatrienal um, während andere Isolate der gleichen Art oder von *S. costatum* nur C7- und C8-Aldehyde bilden. Die meisten der untersuchten Diatomeenarten bilden allerdings überhaupt keine PUA. Entweder ist die Freisetzung der Polyenfettsäuren aus den Komplexlipiden (Berge *et al.* 1995) bei den Produzenten im Vergleich zu den PUA-defizienten Arten unterschiedlich reguliert oder die biosynthetisch nachfolgenden Enzyme sind nicht vorhanden bzw. unterscheiden sich in ihrer Kompartimierung oder Substratspezifität. Putative Gene der in der Katalyse beteiligten Enzyme (= Phospholipase A, Lipoxygenasen) wurden jedenfalls im sequenzierten Genom der PUA defizienten Art *Thalassiosira pseudonana* identifiziert („The Diatom EST Databases" http://avesthagen.sznbowler.com).

d'Ippolito *et al.* (2004) haben Galaktolipide bei *S. costatum* als die wichtigsten Speicherlipide von C16- und C20-Polyenfettsäuren identifiziert und eine Galaktolipase als initiierendes Enzym postuliert. Demgegenüber steht die durch Phospholipase aktivierte Bildung von Decadienal und Decatrienal aus C20-Polyenfettsäuren bei *T. rotula* (Pohnert 2002).

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[12] **VOC**: (= volatile organic compounds). Gemäß der EU-Lösungsmittelrichtlinie sind VOC organische Substanzen mit einem Dampfdruck ≥ 0,01 kPa bei 293,15 K

Da EPA sowohl in Galaktolipiden als auch in Phospholipiden gleichermaßen verteilt vorliegt, die Fettsäuren 16:3 n-4 und 16:4 n-1 aber vorzugsweise in Galaktolipiden gespeichert werden (Yongmanitchai & Ward 1993, Berge *et al.* 1995), kann man eine Regulation der Produktbildung über die Kompartimierung von Speicherlipiden und unterschiedlichen Enzymaktivitäten (z.B.: Lipasen) vermuten.

4.3 Gebiet und Zeitraum der Freilandversuche

In Gewässern der polaren und temperierten Küstengebiete mit einer geringen Stratifizierung (Laabir *et al.* 1998) und einer hohen Durchmischung des Wassers basieren Nahrungsketten auf den dominanten Organismen des Mikroplanktons (Wafar *et al.* 1983, Munn 2004). Solche Gewässer wurden im Englischen Kanal (Roscoff, Frankreich), in der Dabob Bay (Washington, USA) sowie in der Nord-Adria (Italien) untersucht. Sie sind von oligotrophen Gewässern abzugrenzen, in denen der Einfluss von Piko- und Nanoplankton zunimmt. Große Gebiete des tropischen und subtropischen Ozeans haben einen stark stratifizierten Wasserkörper mit einem konstant niedrigen Fluss an Nährstoffen, in denen die Nahrungsketten durch den „*microbial loop*" dominiert werden (Munn 2004). Diese Gebiete waren nicht Gegenstand der vorliegenden Untersuchung.

Die saisonale Abfolge einer Diatomeenblüte mit einem „Klarwasserstadium" beruht nicht auf einem einfachen Massenwechsel, das heißt das Maximum der Phytoplanktonblüte fällt nicht mit einem Anstieg der Kopepodenpopulation zusammen (Smetacek 1975, Lampert & Schober 1978). Der größte Teil der Algenpopulation wird nicht durch Zooplanktonfraß dezimiert, sondern sinkt in tiefere Schichten ab (Drebes 1974, Smetacek 1985) und wird mikrobiell auf dem Weg nach unten in seine organischen und anorganischen Bestandteile zersetzt. Der klassische Erklärungsversuch für dieses Phänomen verweist auf die geringe Populationsdichte der Kopepoden, so dass der Fraßdruck auf die Algenblüte gering ausfällt (Cushing 1975). Während der Diatomeenblüte in der Nord-Adria werden im Vergleich zur dinoflagellatenreichen Perioden gehäuft Missbildungen unter den vorhandenen Kopepodenlarven festgestellt. Es lässt sich daher spekulieren, dass Kopepoden, die sich unabhängig von den Frühjahrsblüten entwickeln können, eine höhere Fitness aufweisen. Daher wurde in dieser Arbeit die Hypothese getestet (**Publikation 5**), ob der niedrigere Fraßdruck während der Frühjahrsblüte unter dem Aspekt einer langfristigen evolutionären Adaption zu verstehen ist.

Viele Studien haben den Untersuchungszeitraum, um den Einfluss der Phytoplanktonblüte auf den Reproduktionserfolg von Fraßfeinden zu studieren, auf das Frühjahr und den Sommer eingegrenzt (Arendt *et al.* 2005, Devreker *et al.* 2005, Maps *et al.* 2005, Jonasdottir *et al.* 2006). Die Zusammensetzung des Phytoplanktons unterscheidet sich aber gerade im Sommer und im Herbst besonders deutlich von der Phytoplanktonsukzession der ersten

Jahreshälfte. Unter der Annahme das Metaboliten unabhängig von der Jahreszeit den gleichen Einfluss auf den Reproduktionserfolg von Fraßfeinden haben, ist eine Beobachtung von biologischen und chemischen Parametern bis November sinnvoll (**Publikationen 12, 14**). Die Verlängerung des Beobachtungszeitraums ist von großer Bedeutung, da z.B.: Kopepoden der Art *Calanus* das ganze Jahr präsent sind, wenngleich die Fekundität temperaturbedingt im Jahresgang rückläufig ist (Bonnet *et al.* 2005; **Publikation 12**).

4.4 Bildung α,β,γ,δ-ungesättiger Aldehyde während der Phytoplanktonsukzession

Die untersuchten Diatomeen-Frühjahrsblüten werden von Arten aus der Familie der Thalassiosiraceae (*S. costatum* und/oder *T. rotula*) dominiert. Zusammensetzung sowie Ausprägung der Blüte unterscheiden sich zwar in den beprobten Gewässern, dennoch sind die meisten blütenbildende Arten, die isoliert und kultiviert worden sind (*T. rotula* in Roscoff, *T. pacifica* in der Dabob Bay und *S. costatum* in der Nord-Adria), starke Produzenten von PUA (Gesamtgehalt: 2-10 fmol Zelle^{-1}) (**Publikation 2, 3, 14**).
Die durch Zellschädigung enzymatisch aktivierte PUA-Bildung wurde somit erstmalig im angereicherten Phytoplankton (Zellgröße > 10 µm) aus dem Küstengewässer vor Roscoff nachgewiesen und eindeutig auf Organismen des Phytoplanktons zurückgeführt (**Publikationen 2, 14**). Im angereicherten Phytoplankton des Küstengewässers vor Roscoff ließ sich von März bis November 2004 das Auftreten von *T. rotula* sehr gut mit dem Gesamtgehalt an PUA korrelieren. Wie in Diatomeenkulturen beobachtet, werden auch im angereicherten Plankton PUA gebildet. Die Annahme, dass *T. rotula* für die Hauptmenge an PUA-Produktion im Plankton verantwortlich ist, wurde abgesichert, indem ein Isolat aus dem Phytoplankton gewonnen und eine Kultur aus einem einzelnen Zellfaden herangezogen wurde. Diese weist dasselbe PUA-Spektrum, wie die Phytoplanktonprobe im Frühjahr auf. Tatsächlich bilden die der Frühjahrsblüte nachfolgenden und ebenfalls isolierten Arten im Frühsommer (*R. setigera*) und Sommer (*G. delicatula*) sowohl *in situ* als auch in Kultur keine oder nur sehr geringe Mengen an PUA. Erst im Herbst mit der Rückkehr von *T. rotula* sind wieder größere Mengen an PUA im Phytoplankton nachweisbar. *T. rotula* kann daher als Biomarker der PUA-Bildung im Küstenbereich von Roscoff verwendet werden. Interessanterweise produziert dieses Isolat aber nur C7- bzw. C8-ungesättigte Aldehyde und ist ein weiteres Beispiel für die artspezifische Varianz der PUA-Produktion (Pohnert *et al.* 2002, Publikation **2**, **3**).
Die PUA-Produktion von *T. rotula* scheint *in situ* wesentlich höher zu sein als in Kulturhaltung (**Publikationen 2**). Unter Annahme, dass nur *T. rotula* Zellen zur Gesamtproduktion an PUA im Phytoplankton beitragen, ist die *in situ* PUA-Produktion um den vierfachen Wert erhöht. Daher bedarf es weiterer Untersuchungen inwiefern biotische

oder abiotische Faktoren bzw. unterschiedliche physiologische Zellzustände, wie sie in den verschiedenen Wachstumsphasen oder beim Zusammenbruch einer Blüte beobachtet werden, den PUA-Gehalt beeinflussen. Die Gehalte an EPA und der Fettsäure 16:3 n-4 korrelieren jedenfalls positiv mit dem PUA-Gehalt im Phytoplankton und sind damit möglicherweise limitierend für die PUA-Produktion. Es fällt auf, dass der Gehalt an EPA und ihrer biosynthetischen Vorläufermoleküle (18:4 n-3, 20:3 n-3; Domergue *et al.* 2003) in Abhängigkeit vom Kulturalter und insbesondere vom Phosphatgehalt im Kulturmedium variiert. Mit steigendem Alter einer Diatomeenkultur nimmt beispielsweise der Anteil von EPA am Gesamtfettsäuregehalt zu, wogegen er sich bei Phosphatlimitierung um bis 75 % verringert (Siron *et al.* 1989, Zhukova 2004).

Da sich Frühjahrsblüten und Diatomeenkulturen in ihrem Verlauf ähneln, lassen sie sich möglicherweise im Labor simulieren (Engelke *et al.* 2005). Die Biomasse und Photosyntheseaktivität steigen in beiden Situationen schnell an und brechen aufgrund von Nährstoffmangel zusammen. Durch den „*microbial loop*“ dominierte Nahrungsnetze verhalten sich dagegen ähnlich wie Kulturen in einem Gleichgewichtszustand eines Chemostaten (Munn 2004).

Weitere Untersuchungen werden die Abhängigkeit der Aldehydproduktion im Phytoplankton von biotischen und abiotischen Faktoren unter kontrollierten Bedingungen aufklären.

4.5 Der Einfluss von Diatomeen auf die Fekundität von *Calanus helgolandicus*

Um den Einfluss natürlich angereicherter Diatomeendiäten (*natural diatom assemblage*: Phytoplanktonprobe, Zellgröße > 10 µm) auf den Reproduktionserfolg von *C. helgolandicus* während des Jahresganges zu studieren, wurden biologische Parameter (Fekundität (= Eiablagerate Weibchen^{-1} Tag^{-1}), Schlupferfolg und Larvenentwicklung) und chemische Parameter ($\alpha,\beta,\gamma,\delta$-ungesättigte Aldehyde, Fettsäuregehalt, POC, NOC) im Englischen Kanal in den Jahren 2003 und 2004 wöchentlich untersucht (**Publikationen 12, 14**).

Der Mittelwert der gemessenen *in situ* Fekundität nimmt im Jahresverlauf 2003 und 2004 mit März beginnend stetig ab bis im Herbst die Eiablage der Kopepoden eingestellt wird und *Calanus* bei stark reduziertem Stoffwechsel in einer Diapause überwintert. Im Frühjahr und insbesondere im Sommer war die Fekundität im Vergleich zu andernorts beobachteten Eiablageraten von *Calanus* stark abgesenkt (**Publikation 12**, Irigoien *et al.* 2000, Bonnet *et al.* 2005). Die biotischen bzw. abiotischen Effekte wirken dabei homogen auf die Population der *Calanus*-Weibchen ein, da der Anteil von eierlegenden Weibchen mit der durchschnittlichen Anzahl gelegter Eier pro Weibchen und Probennahme korreliert (**Publikation 14**).

Sowohl natürliche Diatomeengemeinschaften als auch isolierte Spezies, die für die verschiedenen Phasen der Phytoplanktonsukzession charakteristisch sind, wurden in Fütterungsexperimenten auf ihre Auswirkungen auf den Reproduktionserfolg überprüft. Mit diesem Ansatz ist es möglich, den Einfluss einzelner Arten auf die Entwicklung der Kopepoden zu erkennen. Tatsächlich lassen sich artspezifische Effekte auf die Fekundität feststellen. Während unialgale Diäten von *S. costatum* und *T. rotula* die Fekundität positiv beeinflussen, wird die Eiablage durch *R. setigera* und *G. delicatula* bereits nach einer Fütterungsperiode von zwei Tagen signifikant erniedrigt (**Publikation 12**). Sämtliche getesteten Diatomeenbiozönosen in Roscoff reduzierten ebenfalls die Eiablagerate. Zur Überprüfung des Futtereinflusses wurden Austauschexperimente von Kopepodenweibchen zwischen Regionen unterschiedlicher Phytoplanktonzusammensetzung (Küstengewässer vor Roscoff und Plymouth) durchgeführt, um den regionalen Einfluss der Nahrungszusammensetzung auf die Fekundität zu testen. In der Tat legten Kopepodenweibchen französischer Herkunft eine normale Anzahl von Eiern, wenn sie mit Phytoplankton aus den Gewässern vor Plymouth gefüttert wurden, das weder *R. setigera* noch *G. delicatula* enthielt (< 1% Anteil am Gesamtdiatomeengehalt im Phytoplankton). Umgekehrt wurde die Eiablage von Kopepodenweibchen britischer Herkunft im Einfluss des Phytoplanktons aus Roscoff inhibiert (**Publikation 12**). Man kann daher vermuten, dass einzelne Spezies des Phytoplanktons die Kopepodenentwicklung vermutlich stärker prägen können, als man bisher angenommen hat. Da die PUA-Produzenten *T. rotula* und *S. costatum* einen positiven Einfluss auf die Eiablage haben, ist die Inhibierung der Eiablage

nicht auf PUA zurückzuführen (**Publikation 12**). Vielmehr korreliert die Präsenz von *T. rotula* im Phytoplankton positiv mit der *in situ*-Fekundität im Küstengewässer vor Roscoff (**Publikation 14**).

Die Fekundität von *Calanus* wurde in der Vergangenheit unter vielen Aspekten, wie der Abhängigkeit von der Wassertemperatur, der Körpergröße sowie der Ernährung studiert (z.B. Jonasdottir *et al.* 2006). Insbesondere die Bedeutung der Nahrungsquantität, die als Phytoplanktonbiomasse oder Chlorophyllgehalt angegeben wird (Laabir *et al.* 1998, Arendt *et al.* 2005), sowie der Nahrungsqualität werden diskutiert (Jonasdottir 1994, Jonasdottir *et al.* 2002).

In den Gewässern vor Roscoff korrelieren allerdings die klassischen Indikatoren der Nahrungsquantität wie Diatomeendichte, Chl *a*, POC oder PON weder positiv noch negativ mit der Fekundität. Gleiches gilt auch für den Gehalt an ω3-Fettsäuren bzw. dem Verhältnis DHA/EPA, das ein Indikator für die Nahrungsqualität ist (**Publikationen 12, 14**). Im Jahresgang ähnelt die qualitative Fettsäurezusammensetzung der untersuchten Phytoplanktonfraktion (Zellgröße > 10 µm) dem Fettsäureprofil der dominanten Diatomeen (Dunstan *et al.* 1994).

Arendt *et al.* (2005) haben die Abhängigkeit von Biomasse sowie den Einfluss der Fettsäurezusammensetzung auf die Fekundität von *Temora longicornis* in der Nordsee untersucht. Sie beobachteten eine positive Korrelation zwischen Fekundität und der Zelldichte, die hauptsächlich durch Diatomeen und Cilliaten bestimmt wurde. Die Ergebnisse weisen auf eine Nahrungslimitierung im Untersuchungszeitraum hin. Gleiches wurde kürzlich für *C. helgolandicus* in der Nordsee gefunden (Jonasdottir *et al.* 2006). In anderen beprobten Regionen allerdings korreliert die Fekundität häufig nicht mit dem Chlorophyllgehalt, der als Maß für die Phytoplankton-Biomasse verwendet wird (Devreker *et al.* 2005, Halsband-Lenk *et al.* 2005, Maps *et al.* 2005, **Publikation 12**).

Histologische Untersuchungen der Gonadenentwicklung von Kopepoden mit reduzierter Eiablage haben dagegen in Roscoff gezeigt, dass der Grund für die erniedrigte Fekundität die Arretierung der Eizellenentwicklung ist (Ianora *et al.* 1995; persönliche Mitteilung von Dr. Serge A. Poulet). Da die Eiablage durch einen Nahrungswechsel zu dem Dinoflagellat *Prorocentrum minimum* regeneriert werden kann, handelt es sich um eine reversible Inhibierung. Daher muss die Inhibierung der Eiablage auch *in situ* nicht nachhaltig sein. Während der Phytoplanktonsukzession oder durch Drift der Kopepoden kann sich die Nahrungssituation ändern und positive Nahrungseffekte (*T. rotula*, *P. minimum*) können einzelne schädliche Wirkungen (*G. delicatula*) kompensieren. Die Untersuchungen von Gailhard *et al.* (2002) zur Phytoplanktonzusammensetzung entlang der französischen Küste haben gezeigt, dass Dinoflagellate und Diatomeen in den Sommermonaten häufig koexistieren und somit möglicherweise eine günstigere Nahrungssituation bieten, als das

von Diatomeen dominierte Phytoplankton in den Gewässern vor Roscoff. In dieser Weise sind auch die Beobachtungen von Kleppel *et al*. (1991) zu bewerten, die den positiven Einfluss von Mikrozooplankton auf die Fekundität beschreiben.

Die Situation im Plankton ist auch dadurch kompliziert, dass die Reduktion der Fekundität weder auf die Kopepodenspezies *C. helgolandicus* noch ursächlich auf einzelne Diatomeenarten begrenzt ist. Auch Prymnesiophyten (*Pavlova lutherii),* Chlorophyten (*Dunaliella tertiolecta*) (Lacoste *et al.* 2001) und der nicht neurotoxische Dinoflagellat *Alexandrium tamarense* (Ianora *et al.* 2004) sowie der Haptophyt *Phaeocystis* sp. (Turner *et al.* 2002) blockieren die Eiablage von *C. helgolandicus* oder *Temora stylifera*.

Weder die chemischen Parameter PUA bzw. Polyenfettsäuren noch die Indikatoren für die Nahrungsquantität können die Fluktuationen der Fekundität erklären. Vielmehr verursachen bestimmte Diatomeen eine artspezifische Arretierung der Gonadenentwicklung, die zu einer erniedrigten Eiablagerate führt. Weitere Experimente werden auklären, ob die Inhibierung auf einem bis jetzt unbekannten toxischen Metaboliten, einer defizitären Nahrungsquelle oder auf anderen (a)biotischen Faktoren basiert. Durch einen Vergleich des Metabolom von Diatomeenarten, die unterschiedlich die Fekundität beinflussen, ergeben sich möglicherweise Hinweise auf die Artspezifität.

4.6 Der Einfluss von Diatomeen auf die Schlupfrate und die Larvenentwicklung von *Calanus helgolandicus*

Die Reduktion der Schlupfrate sowie Missbildungen während der Larvenentwicklung bei Kopepoden wurden bis jetzt nur bei einer diatomeenreichen Ernährung gefunden (Poulet *et al.* 1994, Ban *et al.* 2000, Ianora *et al.* 2003). Unialgale Diäten von *S. costatum* (CCMP 2092) oder *T. rotula* (CCMP 1647) erniedrigen die Schlupfrate von *C. helgolandicus* (Poulet *et al.* 1994, Miralto *et al.* 1999a, d'Ippolito *et al.* 2002b, Pohnert *et al.* 2002). Darüber hinaus wurden sowohl in Labor als auch in Freilandversuchen häufig Missbildungen bei den geschlüpften Larven beobachtet (**Publikation 5, 6**). Ein Nahrungswechsel zu dem Dinoflagellat *P. minimum* nach vorangegangenem Frass auf diatomeenreicher Diät erhöht die Schlupfrate bereits nach einem Tag wieder (**Publikation 14**). Um zu überprüfen, ob die von Miralto *et al.* (1999a) vorgeschlagenen α,β,γ,δ-ungesättigten Aldehyde die Missbildungen bei der Larvenentwicklung hervorrufen können, wurde *P. minimum* mit PUA imprägniert. In der Tat rufen die mit Decadienal imprägnierten Dinoflagellaten die gleichen Missbildungen bei Kopepodenlarven hervor, wie die mit der Nahrung aufgenommenen Aldehyd-produzierenden Diatomeen. Eine dinoflagellatenreiche Diät während der larvalen Entwicklung kann die teratogenen Schädigungen nicht mehr vollständig lindern (**Publikation 5**).

Diese durch die maternale Ernährung hervorgerufenen teratogenen Effekte werden allerdings nicht von allen Diatomeenarten hervorgerufen, wie in der Arbeit von Ban *et al.* (1997) berichtet wurde. Da verschiedene Diatomeenarten unterschiedlich die Fekundität, den Schlupferfolg oder die Larvenentwicklung beeinflussen, lassen sich schädliche Einflüsse wahrscheinlich nicht auf einen Parameter reduzieren, sondern sind vielfältiger Natur. Daher ist für eine differenzierte Betrachtung der Ontogenese von der Eiablage, über das Schlüpfen der Larven bis hin zu ihrer Entwicklung genauso notwendig, wie die begleitende Bestimmung des PUA-Potenzials[13] im Phytoplankton (**Publikationen 3, 12, 14**). Während die Fekundität bei unseren Untersuchungen im Englischen Kanal im Frühjahr ihren Maximalwert erreichte und im Jahresgang stetig abnahm, lag die Schlupfrate mit einer Ausnahme permanent unter einer Rate von 80 %. Sie war allerdings in den Sommermonaten signifikant gegenüber dem Herbst erhöht. Unter Berücksichtigung, dass über einem Schwellenwert von 80 % der Schlupferfolg positiv für den Reproduktionserfolg bei *Calanus* bewertet wird (Mauchline 1998, Jonasdottir *et al.* 2006), erscheinen die Bedingungen in dem Untersuchungsgewässer von Roscoff als ungünstig.

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[13] **PUA-Potenzial** = maximal mögliche Menge an α,β,γ,δ-ungesättigten Aldehyden, die von Diatomeen nach Zellverletzung freigesetzt wird [fmol/Zelle].

Die Kombination von niedrigem Schlupferfolg (Frühjahr) und abgesenkter Fekundität (Sommer) sowie die Regeneration der Reproduktion durch einen Nahrunsgwechsel zu *P. minimum* verdeutlichen eindrucksvoll den schädlichen *in situ*-Einfluss des Phytoplanktons auf den Reproduktionserfolg.

Aufgrund der geringen Fekundität und/oder des geringen Schlupferfolges standen für eine statistische Auswertung der Larvenentwicklung zu wenige Individuen zur Verfügung. Weder während des Jahresgangs noch innerhalb kurzer jahreszeitlicher Perioden ließ sich die *in situ* gemessene Aldehydkonzentration mit dem Schlupferfolg korrelieren. Der Schlupferfolg variierte stark und erreichte im Frühjahr einen Maximalwert von 85 % genau zum Zeitpunkt höchster PUA-Produktion (10 fmol Zelle^{-1}) während der *T. rotula* Blüte. Unter der Annahme einer verzögerten Wirkung von PUA ist die der *T. rotula*-Blüte nachfolgende Phase, die nicht durch PUA-Produzenten geprägt ist, von besonderem Interesse. Da in Laborexperimenten der Schlupferfolg innerhalb von zwei Tagen nach einem Nahrungswechsel wieder hohe Werte erreicht (**Publikation 12, 14**) lässt sich der im Freiland ausbleibende Schlupferfolg aber nicht durch eine nachhaltige Wirkung von PUA erklären. Um abzuklären, ob der starke PUA-Produzent (*T. rotula*) dennoch für die Reduktion des Schlupferfolges verantwortlich gemacht werden kann, wurde das Isolat der Alge als unialgale Diät verfüttert. Gemäß den von Miralto *et al.* (1999a) und in **Publikation 5** berichteten PUA abhängigen Effekten auf den Reproduktionserfolg von *Calanus* überraschten die Ergebnisse, da weder der Schlupferfolg noch die Larvenentwicklung durch *T. rotula* negativ beeinflusst worden sind. Die Schlupfrate war lediglich am letzten Tag des einwöchigen Fütterungsexperimentes signifikant erniedrigt, womit auch eine erhöhte Rate an Missbildungen bei den geschlüpften Larven einherging.

Zusammenfassend lässt sich sagen, dass der Schlupferfolg und damit die Vitalität gelegter Eier weder im Freilandversuch mit der PUA-Produktion korrelierte, noch sich eine unialgale Diät des isolierten Aldehydproduzenten *T. rotula* auf den Schlupferfolg oder die Larvenentwicklung negativ auswirkte (**Publikation 14**). Der von Miralto *et al.* (1999a) postulierte negative Einfluss von PUA auf den Schlupferfolg wurde im Küstengewässer von Roscoff bei simultaner Messung biologischer und chemischer Parameter also nicht bestätigt, obwohl die gleichen Kopepoden- und Diatomeenarten untersucht worden sind. Die von Jónasdóttir *et al.* (2006) postulierte Abhängigkeit der Schlupfrate von dem Fettsäureverhältnis DHA/EPA eignet sich ebenso wenig zur Erklärung der hohen Variabilität der Schlupfrate im Jahresgang (**Publikation 14**). Daher müssen andere bisher nicht identifizierte Faktoren als PUA und das Verhältnis von DHA/EPA in Betracht gezogen werden, um die ausgeprägte Reduktion des Reproduktionserfolges von *C. helgolandicus* in Roscoff zu erklären.

Grundverschieden zu den Untersuchungen in Roscoff sind die von Halsband-Lenk *et al.* (2005) durchgeführten Freilandversuche zu bewerten, die chemisch-analytisch im Jahr 2003 begleitet wurden (**Publikation 13**). Diese Untersuchungen stellen zum ersten Mal den teratogenen Effekt einer Diatomeenblüte auf die Larvenentwicklung *in situ* dar. Allerdings wurden die ungesättigten Aldehyde nicht im Phytoplankton selbst nachgewiesen, sondern die isolierten blütenbildenden Spezies wurden als starke Aldehyd-Produzenten in Kultur identifiziert (*T. pacifica* 9,8 fmol PUA $Zelle^{-1}$), so dass keine Aussagen über das PUA-Potenzial im Phytoplankton selbst getroffen wurden (Halsband-Lenk *et al.* 2005). Gleichwohl resümiert die Studie, dass Diatomeen den Reproduktionserfolg, insbesondere die Larvenentwicklung, von *P. newmani* negativ beeinflussen, wenn eine hohe Zelldichte (Frühjahrsblüte) an Aldehydproduzenten erreicht wird und nur wenige Nahrungsalternativen vorhanden sind. Die Freilandstudie in der Dabob Bay bestätigte die in der Nord-Adria und in Laborversuchen beobachten Missbildungen der Larvenentwicklung nach PUA-reicher Ernährung (**Publikation 5, 6**). Freilandversuche von Ban *et al.* (2000) in Funka Bay (Hokkaido, Japan) haben bei der gleichen Kopepodenspezies ebenfalls in Zusammenhang mit einer diatomeenreichen Ernährung eine abnormale *in situ* Larvenentwicklung gezeigt.

Die sich widersprechenden Ergebnisse in der Dabob Bay einerseits und die Freiland- und Laborversuche in Roscoff andererseits können unter zahlreichen Aspekten z.B. hinsichtlich selektiver Nahrungsaufnahme, der Bildung von PUA im Kopepodendarm und artspezifischer Detoxifizierung durch die Kopepoden diskutiert werden.

Selektive Nahrungsaufnahme durch Kopepoden wird häufig angeführt, um zu begründen, weshalb Fütterungsexperimente mit komplexen Mischungen verschiedener Plankter (z.B.: Phytoplankton) schwierig zu bewerten und zu vergleichen sind. Diese Interpretationsschwierigkeiten können überwunden werden (Meyer-Harms *et al.* 1999a, Meyer-Harms *et al.* 1999b, Leising *et al.* 2005), indem die *clearance rate* für die verschiedenen Plankter nach der Fütterungsperiode bestimmt wird. Ein anderer Ansatz untersucht mit Hilfe der Rasterelektronenmikroskopie die Fezes. So kann gezeigt werden, ob die Diatomeen tatsächlich aufgenommen und während der Darmpassage verletzt und verdaut werden (Mayzaud 1986, Ban *et al.* 2000, **Publikation 12**). Weitere Methoden wenden spezifische DNA-Primer für verschiedene Organismen oder die Photopigmentanalyse an, um die Nahrungszusammensetzung im Kopepodendarm zu entschlüsseln (Kleppel & Lessard 1992, Nejstgaard *et al.* 2003, Vestheim *et al.* 2005). Im Rahmen dieser Arbeit wurde die Aufnahme und Verletzung der Diatomeenzellen mit rasterelektronenmikroskopischen Untersuchungen der Feces nachgeweisen (**Publikation 12**). Des Weiteren wurde die Aktivierung der enzymatischen Phospholipase/Lipoxygenase/ Hydroperoxid-Lyase Kaskade durch Fütterung von BODIPY-markierten Phospholipiden detektiert (**Publikation 14**). Das pH-Optimum der putativen Lipoxygenasen und der pH-

Wert im Kopepodendarm von *C. helgolandicus* sind ähnlich (Pond *et al.* 1995). Einen ausreichenden Sauerstoffgehalt im Kopepodendarm von *C. helgolandicus* vorrausgesetzt, kann man daher die Transformation von Polyenfettsäuren durch Lipoxygenasen annehmen. Die Untersuchungen haben also gezeigt, dass die Diatomeen von *C. helgolandicus* aufgenommen und verletzt werden, so dass es zu einer endogenen Bildung von PUA im Kopepodendarm kommen kann. Die Phospholipase Aktivität wird entweder von den Diatomeen bereitgestellt oder enstammt aus dem Kopependarm selbst.

Unter der Annahme, dass *Calanus* in der Nord-Adria, in der Dabob Bay und im Englischen Kanal die von Starr *et al.* (1999) in Fütterungsexperimenten bestimmte Anzahl von Diatomeenzellen auch während einer Frühjahrsblüte pro Tag aufnimmt, ist die Belastung an PUA in der Nahrung in den drei untersuchten Gebieten vergleichbar. Die starken Auswirkungen von *S. costatum* und *Thalassiosira pacifica* auf den Schlupferfolg und die Larvenentwicklung korrespondieren aber mit erhöhten PUA/C- Werten in der Nord-Adria und in der Dabob Bay (**Tabelle 3**). Ob das Verhältnis aus PUA und Kohlenstoffgehalt in der Tat mit dem Reproduktionserfolg von *Calanus* korreliert, werden weitere Fütterungsexperimente mit definiertem PUA- und Kohlenstoffgehalt zeigen müssen.

Tabelle 3 Geschätzte maximale tägliche Belastung der Nahrung durch PUA in den untersuchten Gewässern während der Frühjahrsblüte. Die Werte basieren auf den in Kultur gemessenen Mengen an PUA Zelle^{-1} unter der Annahme unselektiver Nahrungsnahme. Der Schlupferfolg (A_{Larven} [%]) und der Anteil überlebender Nauplius Larven, Stadium N_{III}, ist angegeben (N_{III} [%]). Die Tagesbelastung an PUA [pmol/d] beruht auf einer Aufnahmerate von 2200 Zellen h^{-1} von *Thalassiosira* sp. und 8200 Zellen h^{-1} von *Skeletonema costatum* (Starr *et al.* 1999)

Art	Herkunft	Art	PUA Zelle^{-1} [fmol Zelle^{-1}]	PUA [pmol d^{-1}]	PUA/C [ppm]	A_{Larven} [%]	N_{III} [%]
Pseudocalanus newmani	Dabob Bay	*Thalassiosira sp.*	3.8[a]	200	4132 [a]	50[b]	1[b]
Calanus helgolandicus	Englischer Kanal	*T. rotula*	5.7	300	1348	0-85[c]	n.d.
Calanus helgolandicus	Nord-Adria	*S. costatum*	1.5	295	5750	<40	<10[d]

Anmerkung a) Mittelwert der drei Thalassiosira Arten: *T. pacifica*, *T. aestivalis*, *T. nordenskioeldii*. (Publikation 3); b) niedrige Ausschlüpf- und Überlebensrate der Larven korrespondiert mit einem hohen Anteil an Aldehyd-Produzenten unter den identifizierten Diatomeen während einer dreiwöchigen Phase (Halsband-Lenk *et al.* 2005); c) hohe Variabiliät der Schlupfrate unabhängig vom PUA-Gehalt im Phytoplankton; d) Überlebensrate von Nauplius-Larven, die während der Diatomeenblüte gesammelt worden sind.

Man kann darüber hinaus spekulieren, dass manche Kopepodenarten (*C. helgolandicus*; Roscoff) adaptiert sind und über einen entsprechenden Detoxifizierungsmechanismus für $\alpha,\beta,\gamma,\delta$-ungesättige Aldehyde verfügen (Caldwell *et al.* 2004, **Publikation 1**) oder aber

unbekannte synergistische Effekte notwendig sind (Taylor *et al.* 2005), die zwar zeitweise unter natürlichen Bedingungen gegeben sind, nicht aber unter den in Roscoff durchgeführten Laborexperimenten.

In allen drei untersuchten Regionen beeinträchtigte diatomeenreiche Ernährung den Reproduktionserfolg (*P. newmani* (Halsband-Lenk *et al.* 2005), *C. helgolandicus* (**Publikation 5, 12, 14**), *Metridia pacifica* (Halsband-Lenk 2005)). Die Untersuchungen in der Dabob Bay sowie die Studie von Ban *et al.* (1997) haben illustriert, dass eine diatomeenreiche Ernährung sehr unterschiedlich auf verschiedenen Kopepodenarten wirkt. Tatsächlich zeigte *M. pacifica* in der Dabob Bay eine starke Reduktion der Fekundität, während *P. newmani* Missbildungen in der Larvenentwicklung aufwies (Halsband-Lenk 2005, Halsband-Lenk *et al.* 2005, Leising *et al.* 2005). *C. pacifica* dagegen hat Diatomeen durch selektive Nahrungsaufnahme gemieden und verringerte somit möglicherweise die Belastung an PUA. Die Austauschexperimente von *C. helgolandicus* zwischen Plymouth und Roscoff sowie der Vergleich der Reproduktionserfolge zwischen der Nord-Adria und dem Englischen Kanal haben aber auch gezeigt, dass eine Art sehr variabel auf unterschiedliche Nahrungsquellen reagieren kann.

Im Hinblick auf den methodischen Ansatz ist es erwähnenswert, dass die Untersuchungen in der Dabob Bay und in der Nord-Adria auf den Beobachtungszeitraum im Frühjahr beschränkt waren. Aufgrund der vermutlich hohen Variabilität der PUA-Produktion zwischen Frühjahr und Sommer ist die Sommerblüte aber eine hervorragende Kontrolle, um die Effekte von PUA auf den Reproduktionserfolg zu verstehen und sollte in Studien miteinbezogen werden.

Neben der Funktion als chemischer Verteidigungsmetabolit werden weitere ökologisch relevante Wirkungen von PUA in aquatischen Ökosystemen diskutiert. Jüttner (2005) beschreibt die Signalwirkung von PUA benthischer Diatomeen als Fraßrepellens gegenüber limnischen Kopepoden. Cassotti *et al.* (2005) fand einen wachtumsinhibierenden Effekt von PUA auf Diatomeenkulturen und postuliert einen allelopathischen Effekt bei der Regulierung der Zelldichte von Diatomeenblüten. Allerdings steht hierbei der Nachweis, dass Diatomeen Aldehyde tatsächlich an die Umgebung abgeben, noch aus. Da PUA nur nach Zellschädigung gebildet werden, wäre es interessant, das Meerwasser während der Blütentermination (= Zelllyse) auf PUA zu analysieren. Des Weiteren werden auch andere Lipoxygenase-vermittelte Oxylipine wie die Hydroxyfettsäuren und ihre Ketodiene sowie die C20-Polyenfettsäuren selbst als potenzielle Verteidigungsmetaboliten diskutiert (Jüttner 2001, d'Ippolito *et al.* 2004). Allerdings haben Polyenfettsäuren in den etablierten biologischen Testsystemen mit Seeigel- und Kopepodeneiern keinen Effekt gezeigt (Miralto *et al.* 1999a, Adolph *et al.* 2003, Romano *et al.* 2003; **Publikation 6, 7**).

4.7 Lipoxygenase-vermittelte Biosynthesen bei *Physcomitrella patens*

Für das Verständnis molekularer Wirkungsweisen in trophischen Interaktionen sind die Biosynthese und die Regulation einer Infochemikalie von ebenso großer Bedeutung wie das Verständnis ihrer biologischen Aktivität selbst. Produktdiversität wird zum einem durch Variationen der Vorläufermoleküle und der Katalyseeigenschaften beteiligter Enzyme sowie durch nachfolgende Modifikationen der Produkte erreicht. Die Lipoxygenase-vermittelten Biosynthesen illustrieren diese Prinzipien sehr gut. Ausgehend von α-Linolensäure werden bei den Blütenpflanzen z.B. die Jasmonate gebildet, während Säugetiere Arachidonsäure in Prostaglandine transformieren. Zum Verständnis der verschiedenen Lipoxygenase-vermittelten Biosynthesen haben die Klonierung und Charakterisierung der beteiligten Enzyme sowie der Vergleiche zwischen denen der Blütenpflanzen und der Tiere beigetragen (Blée 1998b, Mueller 2004). Dagegen waren zu Beginn der vorliegenden Arbeit wenige Kenntnisse über diese Biosynthesewege bei Algen und niederen Pflanzen vorhanden (Dembitsky 1993, Gerwick 1994, Pohnert & Boland 2002). Vorallem das Fehlen etablierter molekularbiologischer Methoden für Diatomeen und Makroalgen erschwert die Untersuchungen der beteiligten Enzyme. Daher hat sich das genetisch modifizierbare Laubmoos *Physcomitrella patens* als idealer Modellorganismus niederer Pflanzen herausgestellt (Schaefer 2002). Da das Substratspektrum im Vergleich zu den Blütenpflanzen um die C20-Polyenfettsäuren erweitert ist, ist das Moos geeignet, die Biosynthesewege der von C18- und C20-Polyenfettsäuren abgeleiteten Stoffwechselprodukte zu untersuchen. Die Eicosanoide werden in Laubmoosen hauptsächlich in Phospholipiden gespeichert (Euler 1987, Mikami & Hartmann 2004), deren Acylester in SN1- oder SN2-Position durch eine Phospholipase PLA_1 oder PLA_2 wie bei den Blütenpflanzen hydrolysiert werden (Wang 2001).

Groenewald *et al.* (1990, 1997) und Ichikawa *et al.* (1984) fanden erste Hinweise, dass Bryophyten Prostaglandin $F_{2\alpha}$ sowie Prostgalandin-ähnliche Substanzen, die sich von der Arachidonsäure ableiten lassen, synthetisieren. Des Weiteren wurden Gensequenzen für die Allenoxid-Synthase (AOS) und die Hydroperoxid-Lyase (HPL) in *P. patens* gefunden (Mikami & Hartmann 2004); aber nicht die entsprechenden Produkte. Der Lipoxygenase/ Hydroperoxid-Lyase-Biosyntheseweg wurde im Rahmen der vorliegenden Arbeit systematisch erforscht, indem zuerst das Profil flüchtiger Substanzen und oxidierter Fettsäuren studiert und anschließend beteiligte Lipoxygenasen/Lyasen kloniert und charakterisiert wurden.

Die flüchtigen Substanzen (*R*)-1-Octen-3-ol, 2-Octen-1-ol, (2*E*)-Nonenal und (2*E*)-Octenal sowie die nichtflüchtigen 11- und 12-Oxosäuren werden aus der Arachidonsäure gebildet. Damit produziert das Laubmoos im Vergleich zu den Blütenpflanzen mit Ausnahme des (2*E*)-Nonenals untypische Metabolite (Phillips *et al.* 1979, **Publikationen 8-10**), die aber aus

den Stoffwechselwegen des Menschen (Glasgow *et al.* 1986), der Diatomen (Pohnert 2000), der Pilze (Wurzenberger & Grosch 1984, 1986) und der Rotalge *Chondrococcus* bekannt sind (Woolard *et al.* 1975). Sowohl die Diatomee *Asterionella formosa*, als auch die menschlichen Leukozytenzellen setzten wie das Laubmoos endogene Arachidonsäure über verschiedene Lipoxygenase-vermittelte Reaktionen in die 12-ODTE und in ein zweites jeweils andersartiges Fragment um. Der Pilz *Psalliota bispora* dagegen unterscheidet sich in seiner Substratspezifität für die Linolsäure gegenüber dem Moos bei möglicherweise identischem katalytischem Mechanismus, da beide Organismen (*R*)-1-Octen-3-ol und eine ω-Oxosäure synthetisieren. Wurzenberger & Grosch (1984,1986) haben bei *P. bispora* eine Hydroperoxid-Lyase Aktivität identifiziert, die (10*S*)-Hydroperoxyoctadeca-8*E*,12*Z*-diensäure in (*R*)-1-Octen-3-ol und 10-Oxo-8*E*-decensäure transformiert.

Die Analyse des Produktspektrums einer von sechs potenziellen Lipoxygenasen, die aus *P. patens* kloniert worden ist, überraschte, da sie außer den zu erwartenden Hydroperoxyfettsäuren und den Ketodienen auch flüchtige Produkte sowie eine ω-Oxosäure synthetisiert (**Publikation 9**). Diese multifunktionale Lipoxygenase (= $PpLOX_{mf}$) bildet erst das Hydroperoxid der Fettsäure und führt nachfolgend einen C-C-Bindungsbruch herbei. Die $PpLOX_{mf}$ akzeptiert hierbei sämtliche endogenen Polyenfettsäuren von *P. patens*. Während Arachidonsäure, Linol- und Linolensäure zu den korrespondierenden Hydroperoxiden umgesetzt werden, wird der C-C-Bindungsbruch nur bei der Arachidonsäure katalysiert. Um einen weiteren Einblick in die Ursachen dieser substratselektiven Hydroperoxid-Lyase-Aktivität zu erhalten, wurden Polyenfettsäuren als Substrat in Enzymtests angeboten, die nicht dem natürlichen Fettsäurespektrum der Laubmoose angehören. Nur wenn das Hydroperoxid in Position n-9, wie bei EPA und DHA, eingeführt wird, erfolgt der C-C-Bindungsbruch (**Abb. 17**). Dass es sich dabei tatsächlich um eine katalytische und nicht um eine autokatalytische Lipidoxidation handelt (Spiteller 2003), zeigt die hohe Regio- und Stereospezifität der Intermediatbildung sowie der hohe Enantiomerenüberschuss (*ee* > 94 %) des (*R*)-1-Octen-3-ols (**Publikation 8**).

Um einen weiteren Einblick in die Biosynthesen fettsäureabgeleiteter Metabolite zu erhalten, wurde eine Hydroperoxid-Lyase aus *P. patens* (PpHPL) kloniert und eine Knockout-Mutante dieses Enzyms hergestellt. Das Wachstumsverhalten und der Phänotyp der Knockout-Mutante waren unauffällig. Während die Knockout-Mutante weiterhin die C8-Alkohole und die 12-ODTE produziert, ist die Bildung des (3*Z*)-Nonenals und der 11-Oxoundeca-5*Z*,8*Z*-diensäure (= 11-OUDE) vollständig eingestellt.

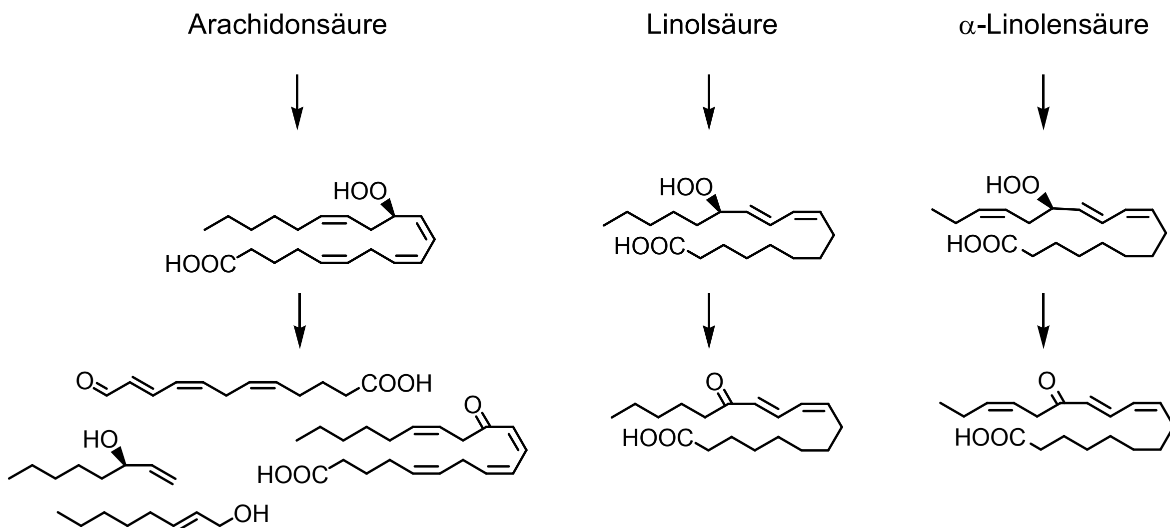


Abb. 17 Die strukturelle Diversität von Oxylipinen einer einzigen Lipoxygenase (LOX_{mf}) wird durch die multifunktionalen Eigenschaften und die vielfältigen akzeptierten Polyenfettsäuren in *P. patens* bedingt. *In vitro*-Transformation der drei häufigsten Polyenfettsäuren in *P. patens*. Dargestellt ist der jeweilige Hauptvertreter unter den Hydroperoxyfettsäuren.

Untersuchungen zu dem Gehalt an Fettsäurehydroperoxiden haben gezeigt, dass das wildtypische Moos hauptsächlich die 12-HpETE und 13-HpOTE produziert. Da die Knockout-Mutanten weiterhin Hexanal bilden können, scheint das bevorzugte endogene Substrat der PpHPL das Hydroperoxid der Arachidonsäure zu sein. Die Arachidonsäure wird somit bevorzugt durch die beiden Lipoxygenase und Lyase vermittelten Reaktionen ($PpLOX_{mf}$ und PpHPL) zu den flüchtigen Substanzen des Moos-Bouquets und den Oxosäuren umgesetzt (**Publikation 10**).

Durch nachfolgende Acetylierungen oder Isomerisierungen der fettsäureabgeleiteten Alkohole und Aldehyde wird das Spektrum zur Verfügung stehender Metabolite auf sehr einfache Weise erweitert. Hildebrand *et al.* (1993) haben bei Tomatenblättern gezeigt, dass fettsäureabgeleitete C6-Alkohole die Fekundität von Blattläusen herabsetzen während (3*Z*)-Hexenylacetat inaktiv ist. Die Acetylierung von (3*Z*)-Hexenol ist somit für die schnelle Inaktivierung von Signalmolekülen von Bedeutung. Interessanterweise haben Toyota *et al.* (1997) 1-Octen-3-yl-acetat im Bouquet verschiedener Lebermoose nachgewiesen, während *P. patens* und einige Pilze den Alkohol als Hauptkomponente freisetzen (**Publikation 8**, Wurzenberger & Grosch 1983). Während *P. patens* den Alkohol nicht modifiziert wird das (3*Z*)-Nonenal durch eine (3*Z*)-(2*E*)-Isomerase zum fungiziden (2*E*)-Nonenal isomerisiert (**Publikation 10**). Eine autokatalytisch Umsetzung von (3*Z*)-Nonenal zu der cytotoxischen Substanz 4-Hydroxy-2*E*-nonenal wurde dagegen nicht beobachtet (Noordermeer *et al.* 2000, Schneider *et al.* 2001). Die Lipoxygenase-vermittelten Biosynthesen von *P. patens* bilden also potenziell biologisch aktive Oxylipine, wenn man entsprechende Beobachtungen bei Blütenpflanzen zugrunde legt.

4.8 Lipoxygenase-vermittelte Biosynthesen bei *Stephanopyxis turris*

Neben enzymspezifischer Substratbandbreite, kaskadenartigen Katalysen können auch spezielle Habitat-bedingte Faktoren das Spektrum Lipoxygenase-vermittelter Biosynthesen bereichern. In diesem Zusammenhang war die Identifizierung von 3-Chloro-1,5-octadien aus der marinen Diatomee *Stephanopyxis turris* interessant, die nach Zellverletzung Chlorid aus dem Meerwasser verwendet, um halogenierte Verbindung zu generieren (**Publikation 11**). Die Alge zeichnet sich durch ihren fruchtigen, angenehmen Geruch aus und unterscheidet sich damit von den anderen untersuchten PUA-Produzenten. Die halogenierte Verbindung hat aufgrund ihrer entständigen *Z*-Butenylgruppe eine strukturelle Verwandtschaft zu den ω3-Fettsäuren, die EPA als Ausgangssubstanz naheliegt. Der hohe Enantiomerenüberschuss von 98 % spricht dabei für eine enzymatische Reaktion. Nach Zugabe von [5,6,8,9,11,12,14,15-$^{2}H_8$]-Arachidonsäure ([$^{2}H_8$]-C20:4ω6) zu einer konzentrierten und verletzten *S. turris*-Kultur, sind die deuterierten Verbindungen [$^{2}H_2$]-3-Chloro-1-octen, [$^{2}H_2$]-1-Chloro-2-octen und [$^{2}H_6$]-12-ODTE nachweisbar (**Publikation 11**). *S. turris* verwirklicht somit einen generell neuen Zugang zu enzymatischen Halogenierungsreaktionen, der sich von den bekannten Mechanismen der Haloperoxidasen oder $FADH_2$ abhängigen Reaktionen unterscheidet (Todd *et al.* 1993, Dembitsky & Srebnik 2002). Außer 3-Chloro-1-octen wurden auch geringe Mengen an 1-Octen-3-ol nachgewiesen, das allerdings als Hydrolyseprodukt der halogenierten Verbindung interpretiert werden könnte.

Für Diatomeen nicht ungewöhnlich, verläuft die Transformation nach Aktivierung der Polyenfettsäuren durch eine Lipoxygenase sehr schnell (Hombeck *et al.* 1999, d'Ippolito *et al.* 2006), so dass das intermediäre Hydroperoxid nur als Hydroxyfettsäure durch Reduktion mit Ebselen (= 2-Phenyl-benzo[d]isoselenazol-3-one) *in situ* abgefangen werden konnte (**Publikation 11**). Auf die LOX-katalysierte Dioxygenasereaktion folgend katalysiert eine Lyase einen Bindungsbruch zwischen C12/C13 und inkorporiert mit hoher Stereospezifität ein Chloridion bzw. Chlorradikal, während *P. patens* eine Hydroxygruppe einbaut. Im Unterschied zur Rotalge *C. hornemanni*, die (*S*)-1-Octen-3-ol synthetisiert, bilden *P. patens*, *P. bispora* und *S. turris* das *R*-Enantiomer des sekundären Alkohols bzw. der halogenierten Verbindung (Woolard *et al.* 1975, Wurzenberger & Grosch 1983, **Publikation 8, 11**).

Entkopplungsexperimente einer Lipoxygenase- und Lyase-Aktivität bei *S. turris* haben gezeigt, dass es sich im Gegensatz zur Katalyse von $PpLOX_{mf}$ vermutlich um verschiedene Enzyme handelt. *S. turris* akzeptiert im Unterschied zur $PpLOX_{mf}$ auch zugesetzte 12-Hydroperoxyeicosa-5*Z*,8*Z*,10*E*,14*Z*-tetraensäure (= 12-HpETE) als Substrat und setzt die Hydroperoxyfettsäure zu den halogenierten Verbindungen und der 12-ODTE um (**Publikation 9, 11**). Vorrausgesetzt, dass der C-C-Bindungsbruch und die Halogenierung

durch das gleiche Enzym katalysiert werden, kann man von einer Halolyase sprechen, deren Isolierung oder Klonierung Einblick in diesen einzigartigen Mechanismus verschaffen sollte.

4.9 Strukturelle Diversität durch vielfältige Reaktionsmechanismen von Lyasen

Die hohe strukturelle Diversität der in dieser Arbeit untersuchten Stoffwechselprodukte beruht auf sehr artspezifischen Reaktionsmechanismen. Während die bis jetzt identifizierten sauren Fragmente eine ω-Oxo-Gruppe aufweisen, besitzt das unpolare Fragment unterschiedliche funktionelle Gruppen (**Abb. 18**). Der Hydroperoxid-Lyase Reaktionsweg der Diatomeen synthetisiert artspezifisch entweder einen Kohlenwasserstoff (*A. formosa, G. parvulum*), einen $\alpha,\beta,\gamma,\delta$-ungesättigen Aldehyd (z.B. *T. rotula*) oder eine halogenierte Verbindung (*S. turris*).

Es ist bekannt, dass Hydroperoxide der Fettsäuren homolytisch z.B. durch Autoxidation oder heterolytisch in einem säurekatalysierten Prozess gespalten werden können (Grosch *et al.* 1981, Schieberle & Grosch 1981, Gardner & Plattner 1984). Für die Katalyse pflanzlicher Hydroperoxid-Lyasen wurde lange Zeit ausschließlich ein heterolytischer Mechanismus angenommen, da das Produktspektrum dem von säurekatalysierten Modellexperimenten entsprach. Hatanaka *et al*. (1986) postulierten einen heterolytischen Mechanismus, weil ^{18}O aus markierten Hydroperoxiden (13-HpOTE) nicht in (3*Z*)-Hexenal, sondern ausschließlich in die 12-Oxo-9*Z*-dodecensäure eingebaut wird. Das Hydroperoxid würde gemäß des postulierten Mechanismus zuerst protoniert und nicht, wie bei Noordermeer *et al.* (2001) diskutiert, in ein Alkoxyradikal transformiert. Unter Wasserverlust (= Verlust eines ^{18}O-Atoms) wird dann ein Epoxycarbokation generiert. Nach Bildung eines allylischen Ethers und Additon von Wasser sowie spontaner Isomerisierung wird (3*Z*)-Hexenal und 12-Oxo-9*Z*-dodecensäure gebildet (**Abb. 10**). Durch die Charakterisierung der HPL als ein Cytochrom P450 Familie wurde allerdings die Annahme eines homolytischen Mechanismus unterstützt. Daher haben Noordermeer *et al.* (2001) den von Hatanaka *et al.* (1986) vorgeschlagenen heterolytischen Bindungsbruchmechanismus variiert, indem sie zuerst die Bildung eines Alkoxyradikals postulieren. Grechkin *et al.* (2004) wiederum haben ein Hemiacetal als das eigentliche Produkt der HPL nachgewiesen, das beide ^{18}O aus markierter [$^{18}O_2$-Hydroperoxy]-13-HpOTE enthält, während Sauerstoff aus [^{18}O]-Wasser nicht eingebaut wurde. Daher schlussfolgern sie, dass die von der HPL katalysierte Umlagerung des Hydroperoxids in ein Hemiacetal homolytisch erfolgt. Dieser Mechanismus lässt sich auf die Bildung von (3*Z*)-Nonenal und 11-OUDE in *P. patens* übertragen (**Abb. 19**).

Für die Bildung von 1-Octen-3-ol kann man in Analogie zu Wurzenberger & Grosch (1986) vorschlagen, dass das Hydroperoxid als Quelle der OH-Gruppe fungiert, die zum C15 transferriert wird. Infolgedessen lagert die Δ^{14}-Doppelbindung um und es kommt zu einem C-C-Bindungsbruch. Das am C12-Atom verbliebene Sauerstoffatom bildet die

Aldehydgruppe des 12-ODTE. Es ist unklar, ob der Mechanismus homolytisch oder heterolytisch verläuft.

Die Verfügbarkeit von Chloridionen im Medium sprechen bei *S. turris* für einen geladenen Übergangszustand und einen nukleophilen Angriff. Die nachfolgende Spaltung der Hydroperoxyfettsäure verläuft wahrscheinlich über einen heterolytischen Bindungsbruch.

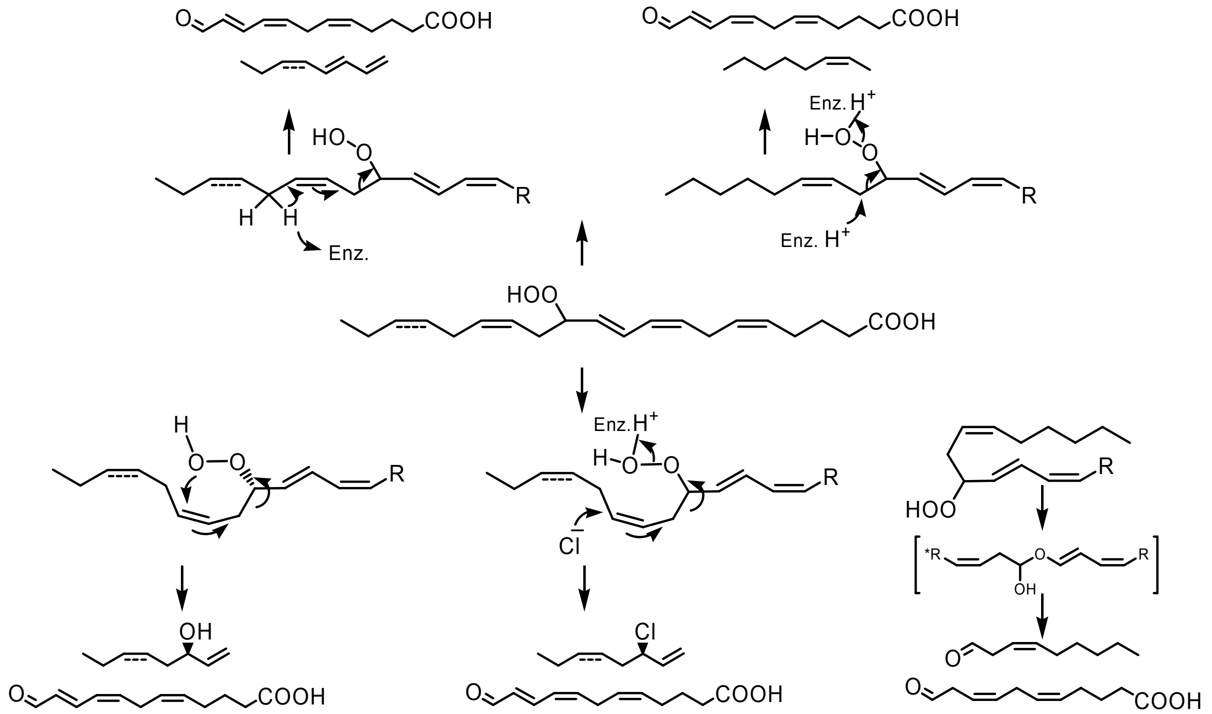


Abb. 18: Verschiedene Hydroperoxid-Lyase-Aktivitäten an 12-HpETE führen zu unterschiedlichen Produkten. Bei allen Umsetzung wird eine ω-Oxosäure und ein zweites Fragment mit variabler Kopfgruppe gebildet. Obere Reihe von links nach rechts: Transformation zu Kohlenwasserstoffen bei *A. formosa* und Säugetieren; untere Reihe: Transformationen bei *P. patens* ($PpLOX_{mf}$), *S. turris* und *P. patens* (PpHPL).

4.10 Vergleich der Oxylipinbildung von Blütenpflanzen und niederen Pflanzen

Bei Blütenpflanzen werden die durch Lipoxygenasen gebildeten Fettsäurehydroperoxide durch mindestens sieben Enzymfamilien weitermetabolisiert (Feussner & Wasternack 2002) (**Abb. 19**). Bislang sind außer Hydroperoxid-Lyasen (HPL), Peroxygenasen (POX), Divinylether-Synthasen (DES) und Allenoxid-Synthasen (AOS) charakterisiert worden (Sembdner & Parthier 1993, Blée 1998a, Itoh & Howe 2001, Noordermeer *et al.* 2001). Hydroperoxid-Reduktasen, Epoxyalkohol-Synthasen (EAS) und ein 9-Hydroxytraumatin bildender Biosyntheseweg sind dagegen bis heute wenig untersucht (Feussner *et al.* 1995, Gardner 1998, Hamberg 1999). Unter Sauerstoffmangel oxidieren Lipoxygenasen ihre Produkte zu den Ketodienen (Kühn *et al.* 1991). Außer durch enzymatische Reaktionen werden Fettsäurehydroperoxide auch autokatalytisch z.B. zu Phytoprostanen oder ungesättigten Aldehyden transformiert (Mueller 2004).
P. patens und die Diatomen nutzen vorzugsweise C20-Polyenfettsäuren zur wundaktivierten Bildung von Oxylipinen und unterscheiden sich damit von den C18-Fettsäure metabolisierenden Blütenpflanzen (**Publikation 8**, Pohnert 2005). Unter den Diatomeen bildet vor allem die Gattung *Skeletonema* eine Ausnahme, da sie hauptsächlich C16-Polyenfettsäuren transformiert (d'Ippolito *et al.* 2004, 2005). Weiterhin haben biosynthetische Untersuchungen zur Oxylipinbildung bei Diatomeen und Moosen gezeigt, dass sich die katalytischen Eigenschaften der involvierten Enzyme ebenfalls von den Blütenpflanzen unterscheiden. Sie ermöglichen damit einen neuen biotechnologischen Zugang zu Duftstoffen (Pohnert 2000, Reski & Frank 2005).

Folgende Lipoxygenase initiierte Transformationen von Polyenfettsäuren wurden im Rahmen dieser Arbeit bei *P. patens* und den Diatomeen untersucht:

- Multifunktionaler-Lipoxygenase-Reaktionsweg
- Hydroperoxid-Lyase-Reaktionsweg (HPL)
- Hydroperoxid-Halolyase-Reaktionsweg

Interessanterweise gehört die $PpLOX_{mf}$, die die erste klonierte Lipoxygenase einer niederen Landpflanze darstellt, basierend auf Sequenz-Homologieuntersuchung zur Gruppe der 13-LOX der Blütenpflanzen. Hinsichtlich ihrer Substratspezifität und katalytischen Eigenschaft ist sie allerdings eher den tierischen oder algalen Lipoxygenasen zuzuordnen (Glasgow *et al.* 1986, Pohnert 2000, 2005, **Publikation 9**). Die klonierte PpHPL zeigt wiederum hohe Sequenzhomologien zu Allenoxid-Synthasen der Blütenpflanzen, doch synthetisiert sie nur geringe Mengen an Produkten des AOS-Reaktionsweges. Da das Enzym vielmehr eine hohe Lyaseaktivität gegenüber verschiedenen Fettsäurehydroperoxiden aufweist, wird es formal als eine unspezifische 9/13 PpHPL klassifiziert (**Publikation 10**). Weitere

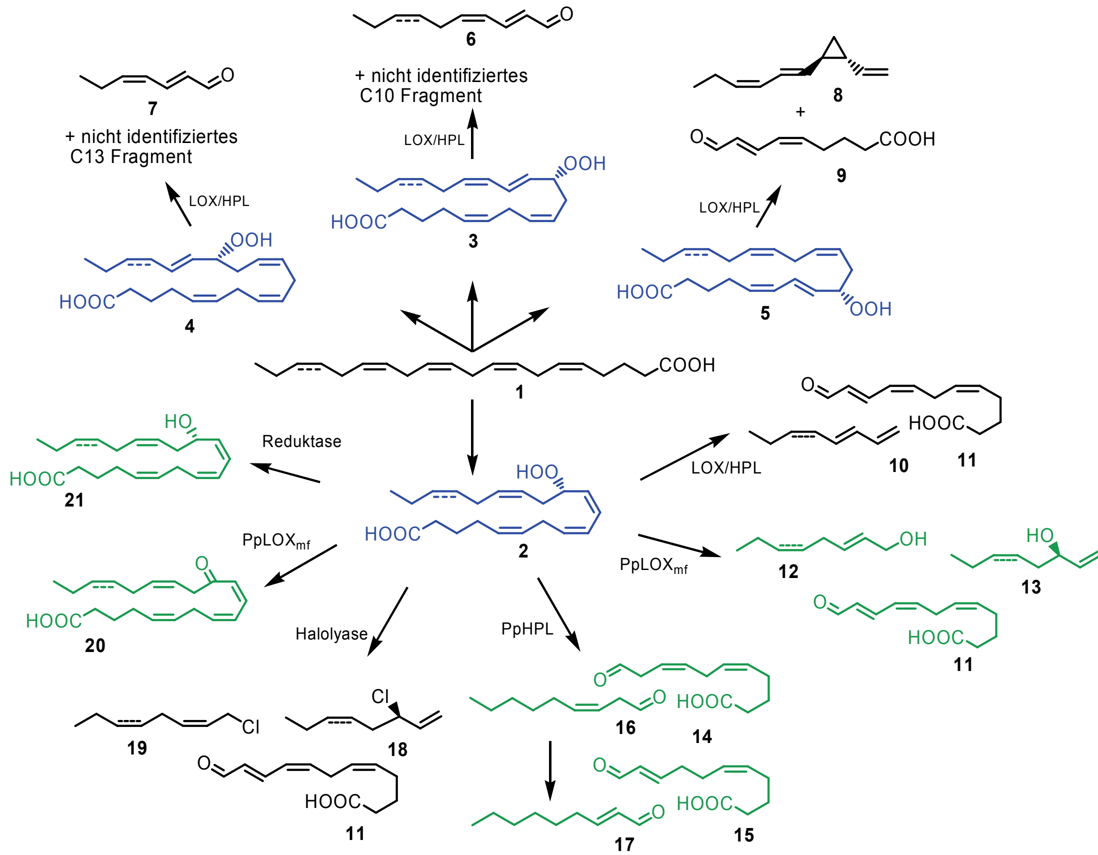
Untersuchungen zur Allenoxid-Synthetase-Aktivität im Moos werden zeigen, ob dieser Biosyntheseweg *in vivo* von Bedeutung ist und ob diese putative AOS-Aktivität wie die Hydroperoxid-Lyase die Arachidonsäurehydroperoxide als Susbstrate *in vivo* präferriert. Die Untersuchungen zur $PpLOX_{mf}$ und PpHPL verdeutlichen, dass der Vergleich genetischer Sequenzhomologien mit denen höherer Pflanzen für eine funktionelle Charakterisierung nicht hinreichend ist, sondern eine Produktuntersuchung erforderlich ist, die sowohl flüchtige als auch polare Oxylipine einbezieht.

P. patens hat sich zu einem interessanten Modellorganismus entwickelt, da er transformiert und genetisch manipuliert werden kann, so dass sich gezielt Biosynthesewege untersuchen lassen. Transformation ist bei Diatomeen mit Ausnahme von *Phaeodactylum tricornutum* derzeit noch nicht etabliert und macht mechanistische Arbeiten damit schwieriger (Montsant *et al.* 2005). *P. patens* ist analog zu den Blütenpflanzen reich an vielfältigen Lipoxygenaseaktivitäten, die direkt oder indirekt in eine chemische Verteidigung eingebunden und die Ursache für die hohe Resistenz gegenüber Schädlingsbefall sein könnten. In diesem Zusammenhang ist es interessant, dass Hamberg *et al.* (2005) kürzlich von der Pathogen-induzierten Genexpression einer α-Dioxygenase bei *P. patens* berichtet hat. Die Entwicklung von biologischen Testsystemen, die die ökologische Relevanz der wundaktivierten Bildung dieser Metabolite untersucht, sollte nun von primärem Interesse sein.

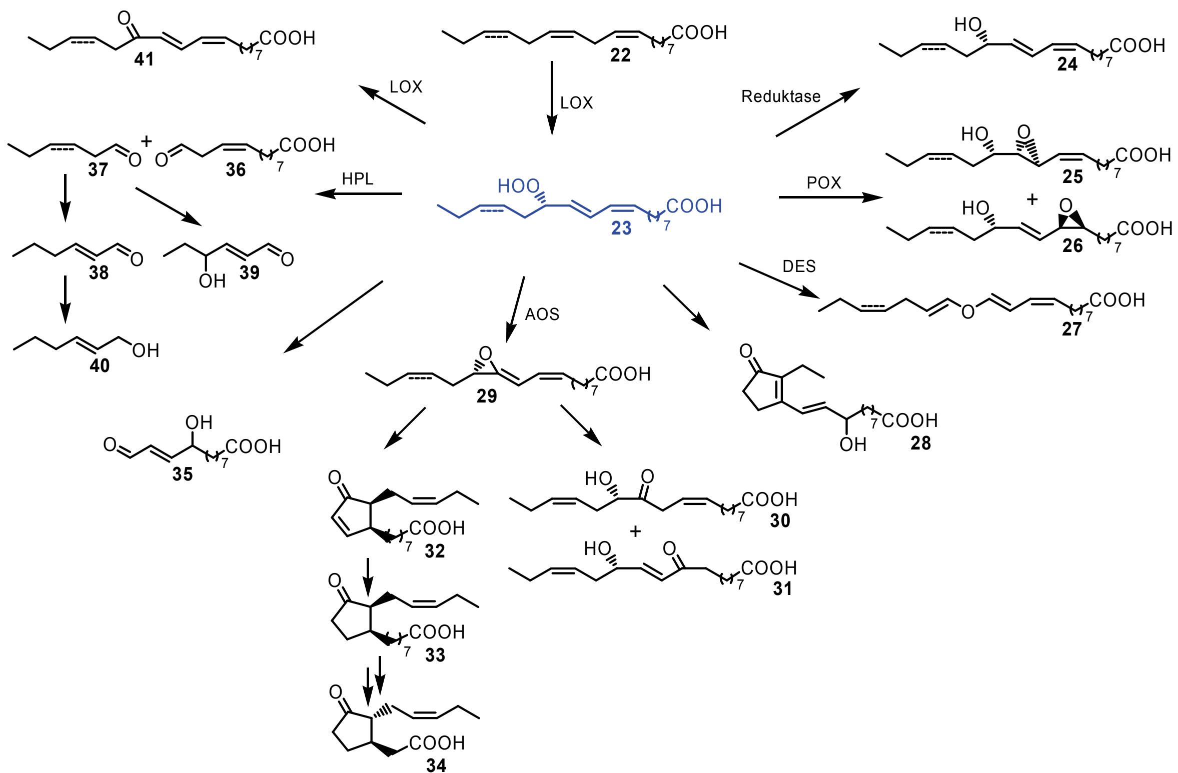
Abb. 19 (nächste Seite): Beispiele von unterschiedlichen Stoffwechselwegen zur Bildung von Oxylipinen der niederen und höheren Pflanzen: (**A**) Umsetzungen von C20-Polyenfettsäuren (**1**) durch Diatomeen und das Laubmoos *P. patens* (grün). Die Hydroperoxyfettsäuren (blau) **3** und **4** können in *T. rotula* auch zu den entsprechenden Hydroxyfettsäuren reduziert (d´Ippolito *et al.* 2006) werden. (**B**) Exemplarische Darstellung der aus 13-HpOTE (**23**) gebildeten Oxylipine bei Blütenpflanzen (nach Feussner & Wasternack (2002) und Schulze *et al.* (2006)). Weitere Positionsisomere der Hydroperoxide von C18-Polyenfettsäuren werden zu einem ähnlich diversen Produktspektrum umgesetzt.

1 Arachidonsäure (ohne gestrichelte Doppelbindung)/Eicosapentaensäure; **2** 12-HpEPE: (12*S*)-Hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaensäure; **3** 11-HpEPE: (11*S*)-Hydroperoxy-5*Z*,8*Z*,12*E*,14*Z*,17*Z*-eicosapentaensäure; **4** 14-HpEPE: (14*S*)-Hydroperoxy-5*Z*,8*Z*,11*Z*,15*E*,17*Z*-eicosapentaensäure; **5** 9-HpEPE: (9*S*)-Hydroperoxy-5*Z*,7*E*,11*Z*,14*Z*,17*Z*-eicosapentaensäure; **6** (2*E*,4*E*,7*E*)-Decatrienal; **7** (2*E*,4*Z*)-Heptadienal; **8** Hormosiren; **9** 9-Oxonona-5*Z*,7*E*-diensäure; **10** (1,3*E*,5*Z*)-Octatrien; **11** 12-ODTE: 12-Oxododeca-5*Z*,8*Z*,10*E*-triensäure; **12** (2*E*,5*Z*)-Octadien-1-ol; **13** (3*R*)-(1,5*Z*)-Octadien-3-ol; **14** 11-Oxoundeca-5*Z*,8*Z*-diensäure; **15** 11-Oxoundeca-5*Z*,9*E*-diensäure; **16** (3*Z*)-Nonenal; **17** (2*E*)-Nonenal; **18** (3*R*)-Chloro-1,5*Z*-octadien; **19** 1-Chloro-2*Z*,5*Z*-octadien; **20** 12-KEPE: 12-Keto-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaensäure; **21** 12-HEPE (12*S*)-Hydroxy-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaensäure; **22** Linolsäure (ohne gestrichelte Line) / α-Linolensäure; **23** 13-HpOTE: (13*S*)-Hydroperoxy-9*Z*,11*E*,15*Z*-octadecatriensäure; **24** 13-HOTE: (13*S*)-Hydroxy-9*Z*,11*E*,15*Z*-octadecatriensäure; **25** 11,12-Epoxy-13*S*-hydroxy-9*E*,15*Z*-octadecadiensäure; **26** 9,10-Epoxy-13*S*-hydroxy-11*E*,15*Z*-octadecadiensäure; **27** Divinylether; **28** Phytoprostan B_1 Typ II; **29** Allenoxid: (13*S*)-12,13-epoxy-9*Z*,11*E*,15*Z*-octatriensäure; **30** α-Ketol: (13*S*)-Hydroxy-12-oxo-9*E*,15*Z*-octadecadiensäure; **31** γ-Ketol: (9*S*)-Hydroxy-12-oxo-10*E*,15*Z*-octadecadiensäure; **32** OPDA: 12-Oxophytodiensäure; **33** OPC-8:0: 3-Oxo-2-[2´(*Z*)-pentenyl]-cyclopentan-1-octansäure; **34** (3*R*,7*R*)-Jasmonsäure; **35** 9-Hydroxy-12-oxo-10*E*-dodecensäure; **36** Traumatinsäure; **37** (3*Z*)-Hexenal; **38** (2*E*)-Hexenal; **39** 4-Hydroxy-2*E*-hexenal; **40** (2*E*)-Hexen-1-ol; **41** 13-KOTE: 13-Keto-9*Z*,11*E*,15*Z*-octadecatriensäure

A) Niedere Pflanzen:
Lipoxygenase vermittelte Bildung von Oxylipinen aus Arachidonsäure/Eicosapentaensäure (**1**)



B) Blütenpflanzen:
Lipoxygenase vermittelte Bildung von Oxylipinen aus Linol-/Linolensäure (**22**)



The research of my thesis stresses aspects of the lipoxygenase-mediated formation of oxylipins that are generally proposed to be involved in chemical defence structuring trophic levels.
In particular, I have focused on diatom/copepod interactions in marine plankton ecology and the role of maternal diets as factor influencing the fecundity, embryonic and early naupliar stages in copepod reproduction. The interactions between phytoplankton and zooplankton are a central topic in plankton ecology. One poorly understood aspect of this interaction is the mass sinking of ungrazed diatom cells at the end of a spring bloom. With diatom diets, copepods had significantly reduced reproductive success compared with other food sources in laboratory experiments. It was hypothesized that the low grazing pressure on blooms is due to the deleterious effect of diatoms on the reproductive success. Others discussed the nutritionally defiencies of mono-diets rather than a toxic effect.
After the initial discovery of the inhibitory effect of diatom-derived $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes (PUA, e.g. 2,4,7-decatrienal or 2,4-octadienal), on the reproductive response of copepods we were motivated to ask for the ecological relevance, the biosynthesis and the abundance of these defence molecules in question. The biological active dienals are formed out of fatty acids by the action of lipoxygenases and fatty acid hydroperoxide-lyases exclusively upon cell disruption.
Taking into account this biosynthesis I have provided improved analytical approaches for *in situ*-derivatisation and quantification of unsaturated aldehydes derived from cultures and phytoplankton samples. For reliable and sensitive aldehyde quantification, diatom containing samples are damaged in the presence of the reagent *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (= PFBHA) under near physiological conditions. This leads to an *in situ* derivatisation without inhibition of aldehyde-biosynthesis.
In order to provide a basis for the interpretation of laboratory and field results I have examined the PUA-production of 51 cultivated or freshly isolated diatom species. Only 38 % of investigated diatoms release PUA ranging from 0.01 to 9.8 fmol cell^{-1}. The screening demonstrates a high species specificity and plasticity of lipid metabolism within the Bacillariophyceae.
In field experiments sampling in coastal waters off Roscoff (Western English Channel, Brittany, France) was performed in 2003/2004. With regard to chemical analysis field studies were also accompanied in the North Adriatic Sea (Italy) and in Dabob Bay (USA) in 2003. In all sampled regions the recruitment rates of copepods derived from diatom enriched diets were temporal less than predicted based on the reproductive responses on non-diatom control food. While in the North Adriatic Sea and in Dabob Bay the reduction of hatchability or larvae malformation coincided with the occurrence of diatoms identified as PUA-producer, evidences are presented in the context of this work shows that the variability of *in situ*

reproductive response (hatchability, fecundity) is not related to *in situ* PUA-production of phytoplankton in coastal waters of Roscoff.

These findings were supported with mono-diets of e.g. T*halassiosira rotula,* where hatching success of "Roscoff copepods" remained high and eggs hatched into healthy nauplii. *T. rotula* was the unique strong PUA-producer (5.8 fmol $cell^{-1}$) during the study period and hence proposed to be a biomarker of PUA. In summary, I conclude that other factors than PUA have to be taken into account in order to explain the temporal reduced reproductive success of *Calanus* in coastal waters off Roscoff.

Several hypotheses have been put forward to explain the suppression of copepod recruitment. Food quantity and quality are major factors influencing growth, reproduction and survival of zooplankton. But, neither the measured parameters chlorophyll *a*, POC and PON indicating the biomass, nor the discussed parameters of food quality C/N ratio and the ratio of ω3-polyunsaturated fatty acids DHA/EPA correlated with any reproductive response factor of *Calanus helgolandicus*. In fact, the reproductive response of *C. helgolandicus* is characterised by a direct relationship between egg production rate and hatchability in coastal waters of Roscoff. This is of interest, because enriched phytoplankton (cell size > 10 µm) containing mainly diatoms impairs dramatically the fecundity.

This work presents evidences that the lipoxygenase mediated pathways in diatoms are very divers. Besides the PUA-producing diatoms and species, which do not release any aldehyde, others transform C20-polyunsaturated fatty acids into acidic unsaturated aldehydes and a further second fragment.

The marine diatom *Stephanopyxis turris* transforms eicosapentanoic acid into halogenated compounds, such as 3-chloro-1,5*Z*-octadiene and 1-chloro-2*Z*,5*Z*-octadiene, and the 12-oxododeca-5*Z*,8*Z*,10*E*-trienoic acid (= 12-ODTE). After introduction of a hydroperoxid functionality into the fatty acid the cleavage is stereoselectively (*ee* > 98%) catalyzed by a newly identified hydroperoxide-halolyase. The understanding of the not yet established enzyme mechanism will presumably challenge the mode of action of known hydroperoxide-lyases.

For further systematic investigations of the wound activated oxylipin formation, we have explored along with Prof. Ivo Feussner (University Göttingen) the release of volatiles of the moss *Physcomitrella patens*. The moss contains high levels of arachidonic acids as well as the typical C18-polyunsaturated fatty acids of higher plants. The oxylipin profile has revealed the preferential transformation of the arachidonic acid. An involved multifunctional lipoxygenase ($PpLOX_{mf}$) and a hydroperoxid-lyase (PpHPL) were cloned and characterized.

While $PpLOX_{mf}$, a novel lipoxygenase with a fatty acid chain cleaving lyase activity, provides (*R*)-1-octen-3-ol (*ee* > 94 %) and (2*Z*)-octen-1-ol and 12-ODTE, PpHPL transforms 12-hydroperoxy- arachidonic acid exclusively into (3*Z*)-nonenal and 11-oxoundeca-5*Z*,8*Z*-

dienoic acid demonstrated by knock out lines. It is interesting to note that the (3*Z*)-nonenal can only trapped by *in situ* derivatisation with PFBHA. In moss it is rapidly transformed by an isomerase activity to the (2*E*)-isomer with increased biological activity.

The unique feature of a plant to integrate foreign DNA sequences at targeted locations by homologous recombination enables the systematic investigation of lipoxygenase mediated pathways using knock-out mutants.

Although the pathology of bryophytes has not yet well developed, *P. patens* became an interesting model system for studies of oxylipins. It is an attractive system for studying the ecological relevance of the proposed infochemicals, because the pathways can be manipulated by knock out mutants.

Im Rahmen meiner Arbeit wurden Aspekte der Lipoxygenase-vermittelten Bildung von Oxylipinen, die als Verteidigungsmetbolite diskutiert werden und vermutlich trophische Interaktionen beeinflussen, untersucht.

Im Speziellen fokussiert diese Arbeit innerhalb der marinen Planktonökologie auf die Interaktion zwischen Diatomeen und Kopepoden. Hierbei wird die Bedeutung maternaler Ernährung als Faktor, der die Fekundität (Eiablagerate), die Embryogenese sowie die frühen Entwicklungsstadien der Nauplius-Larven beeinflusst, genauer diskutiert.

Die Interaktion zwischen Zooplankton und Phytoplankton ist ein zentrales Thema in der Planktonökologie. Ein wenig verstandener Aspekt ist dabei das massenhafte Absinken von nicht gefressenen Diatomeen am Ende einer Frühjahrsblüte. Kopepoden, die sich von uni-algalen Diatomeendiäten ernähern, zeigen einen signifikant reduzierten Reproduktionserfolg im Vergleich zu Nicht-Diatomeendiäten. Daher wurde vermutet, dass der geringe Fraßdruck während der Frühjahrsblüte auf einen schädlichen Diatomeeneffekt auf den Reproduktionserfolg zurückzuführen ist. Andere Hypothesen diskutieren eher die defizitäre Nahrungsqualität von artifiziellen Monodiäten.

Laborexperimente haben den inhibitorischen Effekt von $\alpha,\beta,\gamma,\delta$-ungesättigen Aldehyden (= PUA; wie 2,4,7-Decatrienal oder 2,4-Octadienal), die von Diatomeen gebildet werden, auf den Reproduktionserfolg gezeigt. Diese Ergebnisse motivierten mich die ökologische Relevanz, die Biosynthese sowie die Verbreitung dieser potenziellen Verteidigungsmetbolite zu untersuchen. Biologisch aktive Dienale, die antiproliferativ oder apoptotisch wirken, werden ausschließlich nach Zellverletzung durch die Katalyse von Lipoxygenasen und Hydroperoxid-Lyasen gebildet. Unter diesem Aspekt habe ich eine einfache analytische Methode entwickelt, die eine *in sit*u Derivatisierung und Quantifizierung der gebildeten Aldehyde in Kulturen und im Phytoplankton während Freilandversuchen erlaubt. Dabei wird eine Phytoplanktonprobe unter physiologischen Bedingungen direkt in dem Derivatisierungsreagenz *O*-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamin (= PFBHA) verletzt, so dass die Aldehyde *in situ* ohne Beeinträchtigung ihrer Biosynthese reagieren und stabilisiert werden.

Um einen Überblick über das Potenzial zur Aldehydproduktion innerhalb der Bacillariophyceae zu erhalten, habe ich 51 Arten aus Kultursammlungen und neue Isolate untersucht. Ca. 38 % der getesteten Arten setzen PUA in einem weiten Konzentrationsberereich von 0,01 bis 9,8 fmol PUA Zelle^{-1} frei. Diese Reihenuntersuchung demonstriert die hohe Artspezifität und Plastizität des Lipidmetabolismus.

In Freilandversuche wurden die Küstengewässer vor Roscoff (Westlicher Englischer Kanal, Bretagne, Frankreich) in den Jahren 2003 und 2004 beprobt. Darüber hinaus wurden Feldstudien in der Nord-Adria (Italien) und in der Dabob Bay (Seattle, USA) chemisch analytisch begleitet. In allen beprobten Gewässern war der *in situ* Reproduktionserfolg von

Kopepoden bei einer diatomeenreichen Ernährung im Vergleich zu diatomeenfreien Ernährungen reduziert. In der Nord-Adria und in der Dabob Bay fiel die Reduktion des Schlupferfolgs bzw. das Auftreten mißgebildeter Larven mit dem Erscheinen von Diatomeenarten, die als PUA-Produzenten identifiziert worden sind, zeitlich zusammen. Dagegen werden in dieser Arbeit erste Hinweise präsentiert, dass in den Küstengewässern vor Roscoff die Reduktion des Schlupferfolges von Kopepoden nicht mit der *in situ* Bildung von PUA zusammenhängt. Diese Ergebnisse wurden durch Verfütterung von unialgale Diäten des PUA-Produzenten *T. rotula* abgesichert. *T rotula* wurde als einziger starker Produzent (5,8 fmol PUA Zelle^{-1}) in den Küstengewässern von Roscoff identifiziert und kann daher dort als Biomarker verwendet werden. Trotz der hohen PUA-Produktion schlüpften die Larven und entwickelten sich weitestgehend normal während der Fütterungsdauer. Daraus schlussfolgere ich, dass andere Faktoren als PUA zur Klärung des zeitlich auftretenden eingeschränkten Fortpflanzungserfolges herangezogen werden müssen. Die Quantität und Qualität der Nahrung sind weitere Hauptfaktoren, die das Wachstum, die Reproduktion und das Überleben des Zooplanktons beeinflussen. Nichtsdestoweniger korrelierten weder die Parameter Chlorophyll *a*, POC oder PON, die als Indikator für die Biomasse verwendet werden, noch die bekannten Parameter für die Nahrungsqualität, C/N und das Verhältnis der ω3-Polyenfettsäuren DHA/EPA, mit irgendeinem Fortpflanzungsfaktor von *Calanus helgolandicus*. Der Reproduktionserfolg von *C. helgolandicus* zeichnet sich vielmehr durch eine direkte positive Korrelation zwischen Eiablagerate und Schlupferfolg im Küstengewässer von Roscoff aus. Diese Beobachtung ist von besonderer Bedeutung, da die Ernährung von diatomeenreichem Phytoplankton (Zellgröße > 10 µm) die Eiablage stark beeinträchtigte.

Die vorliegende Arbeit zeigt, dass die Lipoxygenase vermittelten Biosynthesewege in Diatomeen zu einer großen Produktvielfalt führen. Außer PUA-produzierenden Diatomeen sowie Spezies, die keine Aldehyde freisetzen, transformiert eine weitere Gruppe von Diatomeen C20-Polyenfettsäuren zu einer ω-Oxosäure und einem zweiten Spaltprodukt.

Die marine Diatomee *Stephanopyxis turris* bildet aus Eicosapentaensäure die halogenierten Verbindungen (3*R*)-Chloro-1,5*Z*-octadien und 1-Chloro-2*Z*,5*Z*-octadien sowie die 12-Oxododeca-5*Z*,8*Z*,10*E*-triensäure (=12-ODTE). Die Spaltung der Polyenfettsäure wird nach Einführung eines Hydroperoxids durch eine einzigartige Hydroperoxid-Halolyase-Aktivität stereoselektiv (*ee* > 98 %) katalysiert. Die Isolierung dieses Enzyms wird einen neuen Beitrag zum Verständnis der bekannten Hydroperoxid-Lyasen leisten.

Für weitere systematische Untersuchungen zur wundaktivierten Bildung von Oxylipinen haben wir zusammen mit der Arbeitsgruppe von Prof. Ivo Feussner (Universität Göttingen) die vom Modellorganismus *Physcomitrella patens* freigesetzten flüchtige Substanzen analysiert.

Dieses Moos enthält einen hohen Anteil der C20-Polyenfettsäure Arachidonsäure sowie die für höhere Pflanzen typischen C18-Polyenfettsäuren. Die Untersuchung des Oxylipinprofils hat gezeigt, dass im Wesentlichen Arachidonsäure in flüchtige Substanzen und ω-Oxosäuren umgesetzt wird. Daraufhin haben wir eine multifunktionale Lipoxygenase ($PpLOX_{mf}$) und Hydroperoxid-Lyase (PpHPL) kloniert und charakterisiert. Während die $PpLOX_{mf}$, ein neuer Lipoxygenase-Typ mit Lyaseaktivität, (*R*)-1-octen-3-ol (*ee* > 94 %) und (2*Z*)-octen-1-ol sowie 12-ODTE aus Arachidonsäure bildet, transformiert die PpHPL vorzugsweise das 12-Hydroperoxid der Arachidonsäure in (3*Z*)-Nonenal und (5*Z*,8*Z*)-11-Oxoundeca-5,8-diensäure. Interessanterweise konnte (3*Z*)-Nonenal nur durch eine *in situ* Derivatisierung mit PFBHA abgefangen werden, da es sofort durch eine Isomerase zu dem biologisch aktiveren (2*E*)-Nonenal metabolisiert wird. Die einzigartige Eigenschaft des Mooses, Fremd-DNA genortspezifisch über homologe Rekombination zu integrieren, ermöglichte die Manipulation dieser Biosynthesewege. Knock-out-Serien der PpHPL führten zur selektiven und vollständigen Abschaltung der Produktion von (3*Z*)-Nonenal und der 11-Oxosäure bei sonst unauffälligem Phänotyp.

Obwohl die Pathologie der Bryophyten bis jetzt nicht weit entwickelt wurde, hat sich *P. patens* als ein interessantes Modelsystem für Studien über die Bildung von Oxylipinen erwiesen. Die Möglichkeit Biosynthesewege durch Knockout-Mutationen zu manipulieren erlaubt die Überprüfung der ökologischen Relevanz von Stoffwechselwegen und ihren Produkten.

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Publikationen in Vorbereitung

1. **Wichard, T.**, Pohnert G. Wound activated depletion of polyunsaturated fatty acids by the rapid biosynthesis of diatom-derived $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes, in Vorbereitung (2006).
2. **Wichard, T.**, Pohnert G. Trapping (3*Z*)-(2*E*)-Isomerisation products in lipoxygenase mediated pathways, in Vorbereitung (2006).

Vorträge

1. Wichard, T. Mechanismen der aktivierten chemischen Verteidigung von Kieselalgen. *29. Fränkisch-Mitteldeutsches Naturstoffchemikertreffen, Würzburg* (2003).
2. Wichard, T. Cloning and characterization of a lipoxygenase from *Physcomitrella patens* as a model system in the chemical defence of diatoms. *Second German Middle East Plant Molecular Biology Meeting, Jena* (2004).
3. Wichard, T. The influence of diatom-derived aldehydes on the reproductive success of copepods: Determination of deleterious unsaturated aldehydes in phytoplankton. *Second IMPRS symposium, Jena* (2005).
4. Wichard, T., Poulet, S. A. & Pohnert, G. Formation and impact of polyunsaturated aldehydes during the phytoplankton sucession. *ASLO Summer Meeting, Santiago de Compostela, Spain* (2005).

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6. Wichard, T. Neue halogenierte Kohlenwasserstoffe aus marinen Kieselalgen. *33. Naturstoffchemikertreffen: Chemie, Biologie, Ökologie, Jena* (2005).

Posterpräsentationen

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2. Wichard, T., Senger, T., Göbel, C., Kunze, S., Feussner, I. & Pohnert, G. Unprecedented oxylipin pathways in the moss *Physcomitrella patens*: Isolation and characterization of a new multifunctional lipoxygenase with lyase cleaving activity. *XVII International Botanical Congress 2005, Vienna, Austria* (2005).
3. Wichard, T., Poulet, S. A. & Pohnert, G. The influence of diatoms on copepod reproduction: Field and laboratory observations. *53. Winter Meeting of the British Society of Phycology, Plymouth, UK* (2006).
4. Wichard, T., Pohnert, G. Bildung halogenierter Kohlenwasserstoffe durch eine Lipoxygenase/Hydroperoxid Halolyase vermittelte Biosynthese in planktonischen Mikroalgen. *18. Irseer Naturstofftage der DECHEMA, Irsee (2006)*

Workshops

1. Colloquium on Diatom-Copepod Interactions, *Benthos Laboratory of the Stazione Zoologica Anton Dohrn on Ischia, Italy, 3 to 6 November 2002*
2. ESF LESC EXPLORATORY WORKSHOP Influence of Phytoplankton on Herbivore Reproductive Success – Impact of Infochemicals and Food Quality? *Roscoff, France, 29 - 31 March 2006*

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller-Universität ist mir bekannt. Die vorliegende Dissertation habe ich selbständig verfasst und keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel benutzt.

Bei der Auswahl und Auswertung des Materials haben mich die in der Danksagung meiner Dissertation genannten Personen unterstützt. Personen, die bei der Anfertigung der Publikationen beteiligt waren, sind am Beginn der Arbeit („Inhaltsangaben zu den Publikationen“) angegeben.

Ich habe nicht die Hilfe eines Promotionsberaters in Anspruch genommen und Dritte haben weder mittelbar noch unmittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, diese Arbeit oder eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

....

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