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Oxylipin production during a mesocosm bloom of *Skeletonema marinoi*

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ABSTRACT

Numerous biological activities such as grazer defense and intraspecific signaling have been described for diatom oxylipins, fatty acid derived secondary metabolites produced by some diatom species. As the function and importance of these compounds are still controversial, the production of a subclass of these molecules, nonvolatile oxylipins, was studied during an induced bloom of *Skeletonema marinoi* (Sarno et Zingone) in a mesocosm setup. Reproductive parameters of one of the main grazers, *Calanus finmarchicus*, were also examined during the bloom. Oxylipins detected during the bloom were the same as those previously described for *S. marinoi* and were detected predominantly in the mesocosm inoculated with this diatom. Reproductive success of *C. finmarchicus* remained unaffected during the course of the bloom. This may have been due to a dilution effect by the availability of alternative suitable prey or to the limited exposure of the copepods to the oxylipins generated during the short bloom. Follow up laboratory studies showed that oxylipin composition changed both when the *S. marinoi* clone used for inoculation was grown in the laboratory and in comparison to the well-studied Adriatic clone of *S. marinoi*. These results highlight the necessity of quantitatively measuring oxylipin concentrations during diatom blooms at sea to be able to correctly evaluate their ecological significance.

1. Introduction

Diatom lipoxygenase products (collectively called oxylipins) have been suggested to serve numerous ecological functions. First proposed as an insidious grazing defense mechanism (Miralto et al., 1999), oxylipins have also been assigned a putative role in allelopathy (Ribalet et al., 2007a, 2008) and cell to cell signaling (Casotti et al., 2005; Vardi et al., 2006, 2008). The proposed defense mechanism is based on the detrimental impact of diatom diets on the reproductive success of calanoid copepods (reviewed by Ianora and Miralto, 2010). A reduction in hatching success of copepod eggs has been observed in laboratory experiments when females feed on diatom cultures (*e.g.* Ban et al., 1997; Ianora and Poulet, 1993) and in the field during diatom blooms (*e.g.* Ianora et al., 2004; Miralto et al., 2003). In contrast, other field studies have shown diatoms to be a suitable prey for supporting copepod growth and reproduction (Irigoien et al., 2002; Koski, 2007), pointing towards a dose-dependent response of copepods to oxylipins in the diet. In addition to reducing the hatching success of copepod eggs, maternal diets of certain diatom species lead to malformations in hatched nauplii such as missing or reduced limbs (Ianora et al., 2004; Poulet et al., 1995) as a consequence of an induced apoptotic process in naupliar tissue (Fontana et al., 2007; Poulet et al., 2003). These deleterious effects of maternal diatom diets have been linked to the production of polyunsaturated aldehydes (PUA) by diatoms showing anti-mitotic activity (Miralto et al., 1999). PUA are a subgroup of oxylipins which are rapidly formed via lipoxygenase pathways from C_{16}- and C_{20}-polyunsaturated fatty acids (PUFA) released from chloroplastic glycolipids and membrane phospholipids upon loss of cell integrity (Cutignano et al., 2006; d'Ippolito et al., 2003, d'Ippolito et al., 2004; Pohnert, 2000). PUA production in diatoms increases under nutrient stress in culture (Ribalet et al., 2007b, 2009) and induces apoptosis-like cell death in the diatom cells themselves (Casotti et al., 2005). Therefore oxylipins have also been suggested to function as signaling molecules under unfavorable conditions such as those encountered at the end of phytoplankton blooms (Casotti et al., 2005). Such a stress signal may be important in shaping phytoplankton population dynamics such as succession and bloom termination (Vardi et al., 2006).

Abbreviations: DHA, docosahexaenoic acid; EPR, egg production rate; FPR, fecal pellet production rate; NVO, nonvolatile oxylipins; PUA, polyunsaturated aldehydes; PUFA, polyunsaturated fatty acids.

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The proposed role of PUA as an insidious defense mechanism against grazers by induction of a teratogenic effect is controversial (Flynn and Irigoien, 2009; Irigoien et al., 2002; Jónasdóttir et al., 1998). Its relevance in natural systems is still debated (Sommer, 2009) because of contradictory results obtained in field studies on the impact of diatoms on copepod reproduction (Irigoien et al., 2002; Pond et al., 1996; Sommer, 2009) and an absence of correlation between copepod reproductive success and PUA production by phytoplankton (Koski et al., 2008). More recently other oxylipins such as hydroxy-acids and epoxyalcohols have been characterized in diatoms (Barreiro et al., 2011; d'Ippolito et al., 2005; Fontana et al., 2007). These nonvolatile oxylipins (NVO) have been found to impair copepod reproduction in the laboratory by inducing apoptosis in nauplii similar to PUA (Fontana et al., 2007; Ianora et al., 2011) and have been linked to reduced hatching success during diatom blooms at sea (Ianora et al., 2008). They may therefore provide an explanation in some cases for the lack of correlation between PUA production and copepod reproductive success. Like PUA (Ribalet et al., 2007b; Vardi et al., 2006), NVO have been suggested to be involved in diatom bloom termination by functioning as a cell death signal under environmental stress (d'Ippolito et al., 2009).

In this study, mesocosms were used to study the production of NVO by a known producer of these metabolites, the marine diatom *Skeletonema marinoi* (formerly known as *S. costatum*, Sarno et al., 2005). In contrast to laboratory experiments with monocultures, the mesocosm setup allowed us to follow the development of the diatom bloom in a close to natural plankton community. The objective was to examine the production of oxylipins during the development and decline of a phytoplankton bloom, as well as to corroborate previous data on the negative effect of NVO on copepod reproductive success in the field (Ianora et al., 2008).

2. Materials and methods

2.1. Mesocosm setup

A mesocosm experiment was carried out from April 14th to 28th, 2008 at the mesocosm facility of the University of Bergen, Norway (www.bio.uib.no/lsf/inst2.html) as described in Barofsky et al. (2010). The mesocosm bags used in this study are mesocosms B, C, and F as described by Jónasdóttir et al. (2011), Koski et al. (2012), and Vidoudez et al. (2011a). These denominations are maintained to facilitate comparisons between the studies. Briefly, transparent polyethylene bags (11 m^3) were immersed in the bay outside Espegrend Marine Biological Field Station (Bergen, Norway) and filled with unfiltered seawater from 4 m depth from just outside the mesocosm bags. Seawater nutrient concentrations were low (<0.15 μmol L^{-1} nitrate, <0.04 μmol L^{-1} phosphate, <0.11 μmol L^{-1} silicate). Mesocosm bag B was enriched with nitrate and phosphate to initial concentrations of 4.21 μmol L^{-1} nitrate and 0.43 μmol L^{-1} phosphate. Mesocosm bag C was enriched to initial concentrations of 4.24 μmol L^{-1} nitrate, 0.36 μmol L^{-1} phosphate, and 3.53 μmol L^{-1} silicate. In mesocosm bag F, an exponentially growing culture of *S. marinoi* was inoculated at ~500 cells mL^{-1} initial concentration in addition to enrichment with nitrate to 4.06 μmol L^{-1}, phosphate to 0.37 μmol L^{-1}, and silicate to 3.35 μmol L^{-1}. The *S. marinoi* culture used for inoculation was the local strain isolated in 2006 and grown in bulk (10 L polyethylene bags) on f/2 medium (Guillard, 1975) on a 14 h:10 h light:dark cycle at 10 °C prior to inoculation.

2.2. S. marinoi cell counts

Concentrations of *S. marinoi* cells in the mesocosm bags were determined daily in triplicate with a CytoBuoy scanning flow cytometer (CytoBuoy, Woerden, Netherlands). Identification of *S. marinoi* was based on particle scan characteristics determined prior to the experiment. The number of cells was estimated following Takabayashi et al. (2006).

2.3. Oxylipin determination

For mesocosm B, phytoplankton samples were analyzed for days 6, 8, 10, and 12 of the mesocosm experiment. Samples from mesocosms C and F were analyzed for oxylipins on days 6–12, except for day 10 which was lost for mesocosm F. A sample from day 4 was analyzed additionally only for mesocosm F.

Phytoplankton samples were collected by filtering 400–1000 mL of mesocosm water collected daily onto 1.0 μm polycarbonate filters (GE Water & Process Technologies, Trevose, PA, USA) by a vacuum pump. Filters were folded into 1.5 mL test tubes (Eppendorf, Hamburg, Germany), frozen in liquid nitrogen, and stored at −80 °C until analysis. Filters were suspended in 1 mL deionized H_2O (Millipore, Billerica, MA, USA) in the test tubes and sonicated for 1 min on ice. Filter material was removed and the cell lysate was extracted for oxylipin analysis according to Fontana et al. (2007). Briefly, 30 min after sonication, 1:1 v:v acetone (J. T. Baker, Deventer, Netherlands) was added to the cell lysate together with 20–30 μg 16-hydroxy-hexadecanoic acid (Sigma-Aldrich, St. Louis, MO, USA) used as an internal standard for quantification. The acetone/water phase was extracted three times with 1:1 v:v CH_2Cl_2 (Carlo Erba, Milan, Italy). The lower organic phase was recovered, dried over Na_2SO_4, filtered, and after removing the solvent under reduced pressure (Büchi Rotavapor R-114, Büchi Laboratory Equipment, Flawil, Switzerland) the organic material was derivatized with ethereal diazomethane. The resulting methyl esters were analyzed for nonvolatile oxylipins (NVO) by a Qtof-*micro* mass spectrometer (Waters SpA, Milan, Italy), equipped with an ESI source (positive mode) and coupled to a Waters Alliance HPLC system (d'Ippolito et al., 2009). Oxylipin concentrations were calculated based on the volume filtered and correspond to the potential production of all *S. marinoi* cells in 1 L of mesocosm water.

A similar protocol was used for the analysis of a laboratory culture (4 replicates) of the *S. marinoi* clone used for inoculation. This was collected by centrifugation (1000 *g*, 10 min, 4 °C) in a cooled centrifuge with a swing-out rotor (DR 15P, Braun Biotechnology International, Allentown, PA, USA) from a culture in stationary phase grown on f/2 medium on a 14 h:10 h light:dark cycle at 10 °C. The obtained pellet was suspended in 1 mL deionized H_2O (g sample)$^{-1}$ before sonication (Gerecht et al., 2011) and extracted as described above. Oxylipin production in this Norwegian clone was compared to oxylipin production in a clone of *S. marinoi* isolated in 1997 from the Northern Adriatic Sea (4 replicates) (Fontana et al., 2007; Gerecht et al., 2011).

2.4. Copepod reproduction experiment

C. finmarchicus females for copepod reproduction experiments were sorted from zooplankton samples collected approximately one nautical mile west of the mesocosm location in the nearby Raunefjord. A plankton net (diameter: 1 m, mesh size: 333 μm) with a 4 L non-filtering cod-end was towed obliquely from 50 to 0 m depth for collection and females sorted immediately under an inverted microscope in a temperature-controlled room (10 °C). For each mesocosm treatment 15 mature females were incubated individually in 50 mL Falcon culture flasks (BD Biosciences, San Jose, CA, USA) kept in a temperature-controlled room at *in situ* temperature (10 °C) and dim light. Females were transferred daily into new flasks with fresh mesocosm water from that day. Eggs as well as fecal pellets produced in 24 h were enumerated under an inverted microscope. Eggs were then left to hatch for 72 h at 10 °C after which they were fixed with 4% formalin before counting hatched nauplii, abnormal nauplii, and non-viable eggs. Abnormal nauplii were separated from normal nauplii based on incompleteness of limbs.